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Review on molecularly imprinted polymers with a focus on their application to the analysis of protein biomarkers

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Abstract

Molecularly imprinted polymers (MIPs) are a type of artificial polymer, which have complementary cavities that are designed to bind a specific target molecule with a high degree of selectivity. Due to their effectiveness and stability, MIPs have found their way into many applications in medicine, chemistry, analysis and sensing fields. One of the most important modern uses of MIPs is the recognition of biological molecules of medical significance, which are called "biomarkers". The use of MIPs enables easy and rapid extraction and detection of these biomarkers from different biological matrices. There are multiple techniques that arose for synthesis of MIPs each with their own set of advantages and drawbacks. In this review, we discuss MIPs in detail including their different types, methods of synthesis, characterisation methods, common challenges, in addition to their applications in different fields with a focus on their use in the analysis of protein biomarkers.

Keywords: Molecularly imprinted polymers, Biomarkers, Sensors, Proteins, Extraction methods, Polymerisation reactions.

Abbreviations

2-VP	2-Vinylpyridine
4-VP	4-Vinylpyridine
AA	Acrylic acid
ACN	Acetonitrile
ADVN	2,2'-Azobis(2,4-dimethylvaleronitrile)
AFP	Alpha fetoprotein
AIBN	2,2'-Azobis(isobutyronitrile)
Am	Acrylamide
AM1	Austin model 1
APS	Ammonium persulphate

APTES	(3-Aminopropyl)triethoxysilane
ATRP	Atom transfer radical precipitation polymerisation
Aβ	Amyloid-beta
BPO	Benzoyl peroxide
BSA	Bovine serum albumin
CA 15-3	Cancer antigen 15-3
CA-125	Cancer antigen-125
CDs	Carbon dots
CEA	Carcinoembryonic antigen
CNTs	Carbon nanotubes
CRP	Controlled/living radical polymerisation
CV	Cyclic voltammetry
DES	Deep eutectic solvents
DMSO	Dimethyl sulfoxide
DPV	Differential pulse voltammetry
DSC	Differential scanning calorimetry
DSSC	Dye sensitized solar cells
DVB	Divinylbenzene
EAMA	N-(2-aminoethyl)methacrylamide
EDOT	3,4-Ethylenedioxythiophene
EGDMA	Ethylene glycol dimethacrylate
EGFR	Epidermal growth factor receptor
EIS	Electro impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EtOH	Ethyl alcohol
FT-IR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
HEMA	(hydroxyethyl)methacrylate

HER2-ECD	Human epidermal growth factor receptor 2-extracellular domain
HPLC-UV	High performance liquid chromatography-ultra violet detection
LC-MS	Liquid chromatography-mass spectrometry
LCST	Low critical solution temperature
LLE	Liquid-Liquid extraction
LOD	Limit of detection
MAA	Methacrylic acid
MALDI-TOF-MS	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MBA	N,N'-methylenediacrylamide
MD	Molecular simulation dynamics software
MeOH	Methyl alcohol
MIPs	Molecularly imprinted polymers
MS	Mass spectrometry
MWCNTs	Multiwalled carbon nanotubes
Myo	Myoglobin
NIPAm	N-isopropylacrylamide
NIPs	Non-imprinted polymers
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
NSE	Neuron specific enolase
PBS	Phosphate buffer saline
PCM	Polarizable continuum computational model
PDA	Poly(dopamine)
PNE	Poly(norepinephrine)
ProGRP	Pro-gastrin-releasing-peptide
PSA	Prostate-specific antigen
PSB	Poly(styrene) beads
QCM	Quartz crystal microbalance

QDs	Quantum dots
RAFT	Reversible addition-fragmentation chain transfer
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SERS	Surface-enhanced Raman spectroscopy
SPE	Solid phase extraction
SPIONS	Superparamagnetic iron oxide nanoparticles
SPR	Surface plasmon resonance
SWV	Square wave voltammetry
TEMED	N,N,N′,N′-Tetramethyl ethylenediamine
TEOS	Tetraethyl orthosilicate
TGA	Thermogravimetric analysis
Trf	Transferrin
VEGF	Vascular endothelial growth factor
α-CDs	Alpha-cyclodextrins

1. Introduction

Molecularly imprinted polymers (MIPs) are a type of polymers synthesized to identify a specific target molecule with high degree of selectivity. The identification of the target molecule resembles a "lock and key" model, in which MIPs act as the lock that is complementary in size, shape and functional groups orientation to the target molecule; the key [1].

In consequence to the various efforts made in the field of molecular imprinting, different types of MIPs were developed depending on the starting materials used in their synthesis or the technique of synthesis itself. For example, we find types like magnetic MIPs, which contain magnetic materials in its core [2,3], and fluorescent MIPs, which contain fluorescent element in their core or structure [4,5]. Most of the alterations to MIPs either in shape or structure are introduced by modifications to their method of synthesis. In addition, different characterisation methods can be used to identify the structure, shape, porosity and functionality of MIPs, all of which are important parameters to determine their usefulness. Nonetheless, the field of MIPs is full of obstacles, which need to be addressed and sought after for possible solutions. In our review, we discuss all these matters in depth and explore different methods and techniques employed in the course of MIPs production.

Since their recent development, MIPs have been widely investigated by researchers for various purposes. MIPs have been applied to extract and analyse different naturally occurring compounds [6–8], drug molecules [9–11], amino acids [12,13] and pesticides [14]. In addition to small molecules, MIPs have been also applied to larger molecules like peptides, proteins

[15–18], and even bacteria and viruses [19–21]. Moreover, MIPs have been integrated with different sensing methods (electrochemical, optical, or surface plasmon resonance, etc.) to produce a "biosensor", which is considered a very useful and handy analytical tool to detect many bioactive compounds [22–25].

MIPs have been applied in a considerable amount of research papers to extract and quantify protein biomarkers. Owing to the advantages offered by MIPs, there is a huge focus on the development of MIPs of comparable selectivity to this of antibodies in order to be applied in the assay of different biomarkers. Herein, we focus on the research work published in the last ten years concerning the use of MIPs in the analysis of protein biomarkers. In addition, we took the liberty to highlight some of the perks and the challenges of some of these methods in our critical review section.

2. **Biomarkers**

2.1. Definition

The term "Biomarker" is short for "biological marker", which can be defined as any substance found in the body (gene, protein or polysaccharide etc.) that can be of medical or biological significance. This substance can be a part of a normal physiological process or a biochemical pathway or be a result of abnormal pathological condition. Pathological conditions can either generate new molecules into the body or cause a significant decrease or increase in naturally occurring molecules in which case their concentration itself is the biomarker. According to this definition, biomarkers are like signals that give us insightful information about what is going on inside the body with minimal interference and less effort. These signals can also be warnings that a medical condition is evolving, which in turn provides us with a good diagnostic tool that enables early treatment of the condition, hence saving lives [26].

2.2. Classification of biomarkers

Biomarkers can be classified in different ways; the first classification is imaging and non-imaging biomarkers, where imaging biomarkers are the ones we get using different imaging techniques such as X-Rays, PET scans, and MRI. Whereas non-imaging biomarkers are those molecules that have biophysical measurable properties that can be found in different body fluids or tissues. The second classification of biomarkers is according to their nature, biomarkers can be genetic such as DNA, RNA or even a specific gene mutation. On the other hand, they can be protein in nature that vary in size between few Daltons to hundred thousand Daltons (e.g. immunoglobulins). They can also be of very specific nature such as polysaccharides, glycans or glycoproteins [27].

Another method of classification is according to their applications; in that sense we have diagnostic biomarkers, that can be used to detect specific diseases, antecedent biomarkers, that provide means of identifying people at high risk of developing a particular disease, disease prognosis biomarkers, which are most commonly used to follow the prognosis of cancers and chronic conditions. Finally, therapeutic drug monitoring biomarkers, which are used to determine if a drug or combination of drugs is useful for the treatment of a specific condition or to determine the need to adjust the drug doses according to the discovered biomarkers [27]

2.3. Potential benefits of biomarker analysis

In addition, to their diagnostic role, biomarkers are very beneficial in the field of pharmacy and drug development where analyzing patients' and volunteers' biomarkers can predict who will respond better to a certain drug from efficacy and safety perspectives, determine the efficiency of newly discovered drugs, and help reduce the expenses and time of clinical trials [28].

Biomarkers also play an important role in studying the effects of environmental pollution. Pollution biomarkers are defined as quantitative measure of the alterations that happen to a biological system compared to its normal status after exposure to a specific pollutant. Exposure to different environmental pollutants such as heavy metals and toxic gases can result in significant changes in certain physiological processes in the body, the early detection of these variations is useful as sensitive warnings of environmental hazards, before they become on a population scale [29]. Pollution biomarkers vary in specificity; some are very specific such as inhibition of aminolevulinic acid dehydratase that is triggered by exposure to lead, and some are not that specific such as DNA damage or mutations, which indicate exposure to many pollutants, however, in this case, different biomarkers profiling can give us the big image of what kind of pollutant is involved [29].

2.4. Pros and cons of biomarkers' analysis

The analysis of biomarkers has many advantages that makes it one of the most important topics of research nowadays. Biomarker research services market size is expected to reach 18.2 billion dollars by 2026, which proves that biomarker research is a promising field of study [30].

Versatility of biomarkers is also a very good advantage; as more than one biomarker may be found for one medical condition, which can give more certainty about the diagnosis. Another advantage is the high selectivity, as most of the biomarkers usually signal a specific condition with a high degree of precision. Other beneficial merits include reproducibility and their presence in different body fluids and tissues, which enables easy extraction with no need for painful procedures. On the other hand, there are some challenges associated with the analysis of biomarkers, where the most common problem is cross-reaction, which mainly happens due to the poor selectivity of the analytical method. Another problem is that some of these biomarkers exist in very low concentration in body fluids that makes their extraction and analysis extremely challenging; this problem urges the development of very sensitive extraction and analytical methods for their detection. Moreover, a common challenge faced in biomarkers discovery is the low number of case studies involved in the early stages of a particular disease, since the early stages are mostly asymptomatic [31,32]

2.5. Common methods of biomarkers' analysis

In order to analyse and quantify different molecular biomarkers in the body, different analytical tools are used, each method offers some advantages and disadvantages that technically depend on the biomarker itself and its concentration in the sample.

The most commonly used method up till now is enzyme-linked immunosorbent assay (ELISA). Despite the fact that ELISA is the most popular technique used up till now, it suffers from some drawbacks including false positive results with the negative controls when the used blocking solution is not effective, or false negative result when the concentration of the target is too low. In addition, ELISA assay usually requires a multistep sample dilution, washing and preparation that allows a wide field of error and also requires skilled personnel to handle the procedure. Furthermore, antibodies are unstable and require very low storage

temperatures, which adversely affects easy application. Sandwich and competitive ELISA types usually offer very high sensitivity, however they are usually very expensive [33,34].

Other methods of biomarkers' analysis include different chromatographic techniques such as liquid chromatography-mass spectrometry (LC-MS), thin layer chromatography, gas chromatography (GC), and gel electrophoresis. Meanwhile, the problem with most chromatographic assays is that they cannot be applied directly to complex sample matrices such as blood or urine, therefore the target biomarker should be extracted first by a convenient way then injected into the chromatographic method for detection and quantification. Among the promising extraction techniques is the use of MIPs as sorbent material for selective extraction of target molecules, MIPs can be tailored to the target biomarker and be utilized to extract it from any complex matrix with high selectivity.

2.6. Medically useful biomarkers

Protein molecules by far are the most common biomarkers, since they are found in plethora in the human body. Different protein molecules, peptides, or single amino acids can signal a variety of diseases or cancer types. Protein biomarkers are also present mostly in relatively high concentration, which makes their extraction and detection more convenient [35]. For example, estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 are types of tissue-based biomarkers that have been used for years to monitor the course of treatment of breast cancer, DX 21-gene panel is also useful to predict the risk of breast cancer recurrence and the potential benefit of chemotherapy [36]. The intracellular coenzymes NAD(P)H and FAD are involved in all intracellular metabolic pathways by oxidation and reduction reactions. Therefore, they are useful biomarkers for detection of metabolic dysfunctions that happen on the intracellular level and are main causes for many diseases such as diabetes. In addition, these coenzymes are fluorescent by nature, therefore they can be easily scanned without the need for sample pretreatment procedures [37].

The identification of specific mutations in certain proteins such as receptors or enzymes can also help to detect or understand the cause of certain diseases or to target therapy to these specific proteins. For example, according to P. Villalobos et al. epidermal growth factor receptor mutations and anaplastic lymphoma kinase enzyme translocations are promising and novel biomarkers for targeted molecular level therapies in a group of patients suffering from lung cancer [38].

3. **Molecularly imprinted polymers**

3.1. Definition and principle

MIPs are a type of biomimetic synthetic polymers that have a structural memory for specific target molecule. MIPs sometimes are referred to as synthetic antibodies, since they can resemble antibodies in their structural recognition capabilities [1].

The process of synthesizing MIPs usually involves four main elements, which integrate to give the ultimate final structure. These elements are the functional monomer, the cross linker, the initiator, and the porogenic solvent.

The core step of successful synthesis of MIPs is to establish a good interaction between the target molecule and the functional monomer, since this interaction is not only useful during synthesis of MIPs, but also during the rebinding of MIPs with the target molecule in different

samples. Therefore, it is important to choose a functional monomer that has complementary functional groups to those in the target molecule. After the formation of the template-monomer adduct, the crosslinking agent acts like a glue or building block that brings the adducts together in a highly cross-linked polymeric structure as shown in **Figure 1**. No doubt that in order to start polymerisation, an initiator should be added to start a free radical reaction. Finally, the porogenic solvent is used to increase the porosity, which in turn increases the surface area of the formed polymer and enables easy access to the binding sites [1].

The type of bond formed between the template and monomer can be of three types; covalent, non-covalent, and semi-covalent. In covalent imprinting, a reversible covalent bond is formed between the monomer and the target, before they are added to the polymerisation reaction mixture. This bond is then severed after polymerisation to remove the template molecules, and upon rebinding with the target, the covalent bond is re-established. Covalent imprinting is superior due to its high stability, which enables the use of different reaction conditions such as high temperatures or strong acidic conditions. However, the formation of covalent linkage is rather slow and requires a lengthy amount of time for template binding and release. On the other hand, non-covalent imprinting involves the use of weaker bonds such as: hydrophobic interactions, electrostatic, ionic, and hydrogen bonds. Non-covalent binding enables the adducts to be formed *in situ* in the reaction mixture and template molecules can be easily removed after polymerisation through the use of a suitable solvent. Non-covalent binding is much easier to achieve and is applicable to a wide range of targets, moreover, target binding and release is relatively fast. However, non-covalent binding also requires a thorough optimisation of reaction conditions to achieve maximum interaction between the target and the monomer. In addition, non-covalent binding requires the use of excess of monomer molecules to favour the formation of the adduct, yet that can result in the formation of non-specific binding sites that in turn reduces the selectivity of the resulting MIPs. A semi-covalent method is basically an intermediate between the two methods in which the template-monomer adduct is formed by a covalent bond with the rebinding utilizing non-covalent interactions. However, this approach is rarely used, since it is difficult to establish and optimise [39,40].

3.2. Pre-polymerisation and optimisation studies

Pre-polymerisation studies refer to a set of experiments done prior to polymerisation to ensure the stability of the template-monomer adduct and study the effects of various physicochemical parameters. In the case of covalent imprinting, these studies aim at finding a suitable monomer that is able to form a stable covalent bond with the template molecule yet can be easily broken after polymerisation. Among the common covalent bonds that are used in this type of imprinting are Schiff base [41], acetals/ketals [42], or boronate esters [43]. Meanwhile, in case of non-covalent imprinting, weaker bonds are involved such as ionic, Van der Waals and hydrogen bonds, which are easier to achieve with various monomers and targets. In both cases of imprinting, various testing methods are applied to the pre-polymerisation mixture (template with monomer and solvent). These tests are usually conducted using spectroscopic techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FT-IR), and ultraviolet (UV)-spectroscopy. Spectra are collected for template alone and for template-monomer adduct after equilibrium and compared. In most cases, a significant difference can be noticed between both spectra that can give useful information about formation of any bonds besides to their strength and stability. An example of applying UV-spectroscopy was introduced by S. Scorrano et al. when they studied the interaction between their template amino acid; Fmoc-3-nitrotyrosine and five

different monomers. The produced spectra from titration of Fmoc-3 nitrotyrosine in acetonitrile solutions of the monomers revealed a strong bond with the template indicated by a hypochromic shift from the original spectrum of the template alone. Therefore, 4-vinylpyridine was chosen in this study, since it had the strongest and most stable interaction [12].

A more in depth prepolymerisation study is to compare different ratios of template to monomer in order to determine the most stable ratio. Using spectroscopic applications such as Job's method, binding isotherm and titration curves can reveal the nature of interaction, association constant (K_a) and coordination number of the complex [44].

Optimisation usually aims at various important goals including: favouring the formation of a stable template-monomer adduct, the formation of a uniform size and porous polymer particles, easy and complete template removal and rebinding, reducing non-specific binding, and maintaining the structural and conformational integrity of the target molecules especially the bulky ones such as proteins. Optimisation of MIPs synthesis involves changing the type and/or concentration of the template, functional monomer, cross linker, initiator and porogenic solvent (classical optimisation). Batch rebinding of template by the produced polymers is used as a measure for the optimum condition. Finally, some papers reported the use of software techniques such as an artificial neural network to carry out optimisation with minimal number of experiments and high accuracy [45].

To elaborate on classical optimisation, we must explain the role of each element in polymerisation and the importance of optimisation regarding the type and concentration.

a) Functional monomer

The choice of monomer mainly depends on the template, where the monomer should have functional groups complementary to those in the template molecule. In the case of covalent imprinting, amides or esters of acrylic acid or methacrylic acid are most frequently employed, where templates are bound to the vinyl moieties of the monomer. However, in the case of non-covalent imprinting, similar vinyl monomers can be used if they contain functional groups that can interact with the template by hydrogen bonds. A very popular monomer is methacrylic acid (MAA), which is considered as a universal monomer due to its hydrogen bond donor and acceptor dual characteristics. Along with MAA, acrylic acid (AA), acrylamide (Am), and vinyl-pyridines (2-VP and 4-VP) are other commonly used monomers which can interact with a wide variety of templates. Structures of these monomers are illustrated in **Figure 2a**.

N-isopropylacrylamide (NIPAm) (**Figure 2a**) is a special type of functional monomer, which has the advantage of thermosensitivity. Poly(NIPAm) polymers and hydrogels undergo volume phase transitions at lower critical temperatures (~32°C). Therefore, polymers made using NIPAm possess a reversible thermal transition response, which is useful to control the capture and release of template. M. Fang et al. prepared a fluorescent polymer using NIPAm. It was found that the polymer binding capacity was significantly higher at 28°C compared to 44°C because the polymer presents a hydrophilic and swelling state at 28°C, which enables easy access of the target molecules to the imprinted cavities [46]. Nonetheless, polymer chains prepared with NIPAm fold randomly during transition from extended coil structure to a collapsed globule structure, therefore the conformation of the newly formed globules might differ from the original one, which in turn can affect the structural selectivity of the polymer [47].

Some researchers reported synthesis of monomers that have a specific functionality to be used in MIPs. For example, W. Xu et al. reported synthesis of an imidazolium-based ionic liquid with vinyl groups that could provide multiple interaction with template molecules and hence increased selectivity [48].

Deep eutectic solvents (DES) are a class of compounds prepared by mixing a hydrogen bond acceptor, mainly a quaternary substituted ammonium salt with a hydrogen bond donor at certain ratios. They resemble ionic liquids in some characteristics such as low melting and boiling points, high conductivity, electrochemical stability and excellent solubility for many substances. However, DES surpass ionic liquids by offering the advantages of ease of preparation, low cost, biodegradability and low toxicity, which make them ecofriendly. Recently, many papers prepared MIPs using DES as functional monomers, which had a significant positive impact on the selectivity, affinity and greenness of the resulting polymers [49–52].

Optimisation of a monomer's concentration is equally important to choosing its type. Association between the monomer and the template is controlled by an equilibrium, therefore it is preferred to add the functional monomer in excess to favour complex formation. However, we need to mention that too much monomer also results in the formation of multiple heterogeneous binding sites, which in turn reduces selectivity. Therefore, it is important to reach the optimum molar ratio, which in fact differs according to the nature of template, so we cannot recommend a specific ratio [53].

b) Cross linker

A cross linker is the backbone of structural rigidity, which in turn allow MIPs to preserve their memory of the template after it is removed. Different cross linkers can result in variable structures of binding sites and different orientation of functional groups available for binding. [54].

As simple as the role of cross linker might appear, its type and concentration should be carefully optimised. Firstly, the chosen cross linker should have a similar reactivity to the functional monomer, this ensures that none of them will polymerise predominantly and that polymerisation is random with uniform distribution of functional residues. Secondly, the concentration of cross linker should not be very low, as this reduces the mechanical stability of the resulting polymers, in addition the binding sites will be located too closely to each other, which will inhibit efficient binding. On the other hand, higher concentration of cross linker reduces the number of binding sites per unit mass of MIPs, besides to the destruction of selectivity in the case of the side non-covalent interactions between cross linker and template or cross linker and the monomer [54].

Common cross linkers employed in MIPs synthesis are N,N'-methylenebisacrylamide (MBA), ethylene glycol dimethacrylate (EGDMA), and divinylbenzene (DVB) structures of which are illustrated in **Figure 2b.** Cross linkers may also be synthesised according to the required functional needs. R. Xu et al. [47] reported the synthesis of a new peptide cross linker that exhibited a pH dependent helix-coil transition properties. Therefore, changing the pH from 5.5 to 7.4 led to expansion of the imprinted cavities and easy template removal; restoring the pH to 5.5 returned the imprinted cavities to their original shape and size due to the precise peptide folding.

Another smart and ecofriendly approach was introduced by Y. Sun et al. [17], where they utilized dopamine as both functional monomer and a cross linker for synthesis of MIPs for

detection of bovine serum albumin (BSA). The idea is based on the self-polymerisation of dopamine in weak alkaline medium (pH 8.5). Therefore, in the presence of template protein in weak alkaline medium, dopamine polymerises around the template creating cavities with high recognition without the need for a cross linking agent.

c) Initiator

Cationic, anionic and free radical polymerisation reactions can all be applied for the synthesis of MIPs. However, free radical polymerisation is the most popular due to its experimental easiness and versatile applications. 2,2'-azobis(isobutyronitrile) (AIBN) and 2,2'-azobis(2,4-dimethylvaleronitrile) (ADVN) (**Figure 2c**) are the most common azo initiators used in free radical polymerisation. The reaction is usually initiated thermally at temperatures ranging from 50 to 70 °C, however this temperature range can be hazardous to the stability of the monomer/template adduct or to the template itself (e.g., proteins). Therefore, photochemical initiation using UV light can be applied in such cases [55]. Some oxidizing agents have been applied as initiators in recent papers, mostly a mixture of a strong oxidizing agent and an accelerator such as potassium persulfate and sodium bisulfite mixture [18] or ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-Tetramethyl ethylenediamine (TEMED) mixture [4,56]. In all cases, dissolved oxygen in the reaction mixture must be removed prior to polymerisation simply by purging inert gas such as nitrogen or helium.

d) Porogenic solvent

The prime roles of porogenic solvent are to solubilize all the starting materials of the reaction and act as a pore forming agent in the resulting MIPs. Solvent molecules are trapped in the polymers and are removed after drying, which leaves behind pores. These pores are beneficial for easy access of template to the binding sites. The polarity of the solvent is an important influential factor, especially in non-covalent imprinting. Solvents should promote the formation of bonds between template and monomer; therefore, it is advisable to use non-polar aprotic solvents. Chloroform, acetonitrile, tetrahydrofuran and toluene are common porogenic solvents used for successful non-covalent imprinting. Water is a very poor choice of solvent in non-covalent imprinting, since it disrupts the hydrogen bonds between template and monomer. However, many templates, especially those of biological interest, are soluble in water only, which present a challenge for non-covalent imprinting of such templates [55].

e) Template

Careful study of the template chemical structure should be taken into account. It is important to ensure the presence of accessible functional groups in the template that are capable of interacting with the functional monomer. Nevertheless, these functional groups should not prevent or interfere with polymerisation reaction itself. Furthermore, the chemical stability of the template should be investigated under the applied reaction conditions (pH, temperature, etc.) to ensure that its structural integrity remains intact during polymerisation [57].

3.3. Theoretical and computational approaches in MIPs design

With the huge progress in the field of computational analysis and statistical software programs, it was only predictable that computational strategies will contribute in the field of MIPs design. Using these technologies, scientists now are able to successfully optimise the procedure for MIPs synthesis with high accuracy and minimal time, effort and cost using a set of sophisticated calculations performed by state-of-the-art software.

Computational methods for the study of electronic structure have been used effectively in prepolymerisation studies to investigate the interactions between the template and functional monomers. Herein, technique such as semi empirical Austin model 1 (AM1) enables the calculation of binding energy between template molecules and potential functional monomers. In addition, the AM1 model allows the optimisation of the template to functional monomer ratio. Since the development of the AM1 model, it has been used in many research papers to guide the process of optimisation and polymerisation of MIPs. Moreover, theoretical data via the software can also be compared to practical results obtained from different characterisation methods such as NMR and FT-IR to verify the usefulness of the computational techniques [58–61]. Nevertheless, a common problem encountered with electronic structure methods is the need to incorporate all the atoms in the prepolymerisation mixture into the computational model, which is very difficult. In addition, the inclusion of a suitable number of the solvent molecules in these calculations makes them more time consuming, so most researchers omit solvent effect totally from their calculations.

The polarizable continuum computational model (PCM) is a successful alternative for studying the solvent effect, PCM approximates the effect of solvent in the experiment by placing the prepolymerisation system in a cavity with a polarizable surface. The polarization is usually dependent on the dielectric constant of the solvent used, which is an easily measured quality and therefore can be included in the calculations. However, PCM has its own limitations with protic solvents, since it fails to include the effect of hydrogen bonds exerted by solvent molecules, which in turn compete with the template-monomer hydrogen bonds [62].

Another useful computational technique is molecular simulation dynamics software (MD). Using this software, we can gain insights about the stoichiometry of different polymer components in addition to the effect of other variables like temperature and pressure. This software creates different simulations to the monomer-template complex in the prepolymerisation mixture and allows for choosing the optimum one. In this way, The MD is able to create a sort of virtual library including huge number of monomers and a simulation of their interactions with the template under different sets of variables. Due to the high accuracy and time saving qualities of MD technique, it is applied specially to study large molecules such as proteins and DNA, which are of particular interest nowadays to be used as templates for MIPs [63,64].

Further insights about the use of computational analysis in the field of MIPs are included in the review by T. Cowen et al. [65].

3.4. Methods of molecular imprinting

Since their development, methods for synthesising MIPs have developed enormously. Bulk molecular imprinting was the first method applied for MIPs. However, due to the many problems associated with bulk imprinting, many other techniques evolved over time each with their own advantages and applications. It is to be noted that in all types of molecular imprinting, non-imprinted polymers (NIPs) are synthesised simultaneously in the absence of template molecules for the purpose of comparison and error elimination.

3.4.1. Polymerisation in one reaction vessel

- Bulk polymerisation

Bulk polymerisation is the simplest and most rapid method of molecular imprinting. In this type of imprinting the template molecules are added to the reaction vessel with the

monomer, cross linker, initiator and usually a small amount of solvent to start the free radical polymerisation. Herein, the template molecules are imprinted as a whole into the 3-dimensional matrix of the polymer. These 3D imprinted cavities allow for high recognition and readily accessible binding sites for the template. After polymerisation, the product is ground and sieved to obtain fine particles [66].

Bulk imprinting is preferred for small template molecules, since they can be easily bound and released from the binding sites in a reversible and reproducible way. In case of large molecules, it would be expected that the imprinting of the whole molecule in the polymer structure will result in increased recognition, which is true. However, some drawbacks are inevitable in the imprinting of large molecules especially proteins.

First of all, the use of grinding results in irregular size distribution of the produced polymer, which reduces their applicability especially for chromatographic purposes. Besides, grinding also causes mechanical disintegration of the intact particles, which in turn produces heterogeneous binding sites and reduces sensitivity dramatically [67]. Secondly, the larger the template molecules, the more time they take to be released or bound to the binding sites, which makes the binding of proteins, microorganisms and DNA highly time consuming [68]. Third, during protein imprinting, problems such as maintaining conformational stability cannot be ignored [66]. Finally, the large imprinted cavities can also attract smaller polypeptides in the sample resulting in cross reactivity and reduced selectivity [68].

Due to the easiness and time saving qualities of this method, it is applied until today for imprinting of drugs [69,70], products from a plant origin [71,72], and proteins [73].

- Suspension polymerisation

In this type of polymerisation, the mixture is mechanically agitated or exposed to ultrasonic waves to be divided into fine droplets in a dispersion medium. Usually, water is used as a dispersion medium, however it is not very favourable, since water disrupts hydrogen bonds in the template-monomer adduct, therefore, mineral oils or perfluorocarbon liquids are more favourable. Suspension polymerisation usually results in spherical, large polymer beads in the micrometre to millimetre range, so it is more beneficial for large scale synthesis of MIPs. The main drawback of suspension polymerisation is that dispersion media might adversely affect the recognition of target molecules [67]. By selecting the suitable dispersion media, suspension polymerisation can be easily applied to a wide variety of drugs, chemicals and pollutants [74,75].

- Emulsion polymerisation

Emulsion polymerisation involves the use of surfactants or cyclodextrins to form a water in oil or oil in water emulsion of the polymerisation mixture. Therefore, the monomer, cross linker and initiator are dissolved in one phase mostly the organic one, and the template with surfactant are dissolved in the opposite phase. The two solutions are then mixed with vigorous shaking. The formed emulsion results in fine mono-dispersed polymer particles with a very small particle size from tens to hundreds of nanometres. After polymerisation, de-emulsification is done by adding an organic solvent such as acetone [68].

The obvious drawback of this method is the use of water and surfactants, since they disrupt the bonds between template and monomer; moreover, surfactants may isolate the template in their core preventing it from being imprinted at all. Nonetheless, emulsion

polymerisation is a popular technique that is still applied up till the present for many templates [76–79]

- Precipitation polymerisation

Precipitation polymerisation is somewhat similar to bulk polymerisation yet superior in performance. This type of polymerisation is carried out with high concentration of template, large volume of solvent and relatively high stirring speeds. Therefore, polymerisation occurs in dilute solution and polymer growth starts with the nanogel seed particles followed by entrapment of larger oligomers. Eventually when the polymer particles exceed a certain size, they start to precipitate from the solution. As a result, spherical particles with uniform particle size are obtained usually with a diameter less that one micrometre. The use of high stirring speeds as well as dilute solutions produce a smaller particle size in comparison to the polymers produced by suspension method. The major drawback of this method is the use of a large volume of solvent, which reduces the greenness of the method in case of using organic solvents. Besides, using higher concentration of template may not be economic for expensive and rare templates [67,80].

There are several factors that need to be carefully considered and optimised during precipitation polymerisation to control the particle size such as: polarity of solvent, stirring speed, concentration of monomers, and reaction temperature. So far, precipitation polymerisation has been successful in imprinting many templates from small molecules [81–83] to large proteins [18]. Moreover, due to the spherical shape and uniform size of the resulting polymers, they are useful as potential stationary phases for chromatographic separations [84].

- Multi-step swelling (seed) polymerisation

Multi-step swelling polymerisation is a sophisticated and time-consuming method used to prepare uniform and spherical MIPs. Polymerisation takes place in gradual steps starting by the use of small polymeric seeds e.g.: polystyrene. Particle swelling increases in each step until MIPs of uniform size are obtained. Despite the prolonged procedure and the need to extensively optimise each step of polymerisation, multi-step polymerisation has few applications with different templates [85,86].

3.4.2. Surface imprinting on a solid substrate

Because of the many drawbacks associated with the bulk imprinting technique, a new technique has surfaced in the 2000's, which is known as surface imprinting. Herein, the binding sites of MIPs are formed on the surface of a solid substrate or around nano/micro sized solid particles. As implied from this description, surface imprinting provides a great advantage namely the readily accessible binding sites, since the target molecules do not have to migrate through the complex structure of MIPs formed by bulk imprinting. Therefore, binding of target is not diffusion limited, which enables easy and rapid binding and release. Consequently, this technique is very popular for imprinting of large molecules and proteins. However, it also suffers from some drawbacks including the smaller number of available binding sites per unit area, which in turn can reduce sensitivity. In addition, the procedure of synthesis is usually time-consuming and includes multiple steps [87,88].

- Soft lithography

In soft lithography, nano/micro scaled patterned MIPs films are formed on solid substrates using a soft polymeric stamp. The procedure is quite simple; the pre-polymerisation mixture is spotted on the surface of a solid substrate usually a glass slide. The solution is then squeezed between the glass slide and soft poly(dimethylsiloxane) mold. Suitable pressure is applied, and UV light is used to initiate the reaction. As a result, thin patterned polymeric films are formed and then dried. After the removal of the template by a suitable solvent, thin layered MIPs films with highly accessible and complementary binding sites are obtained. Moreover, the presence of the stamped patterned network on the MIPs film increases the exposed surface area and hence increases the binding capacity [89,90].

- Core-shell surface imprinting (grafting)

In this type of surface imprinting, MIPs are grafted around nano/micro sized solid particles. In contrast to soft lithography, MIPs are formed on the surface of many small particles instead of a planar solid surface, which immensely increases the exposed surface area. Moreover, the use of solid particles enables accurate control of the MIPs particle size, morphology, and surface area [88]. **Figure 3** illustrates a representation for the core shell imprinting procedure on nanoparticles (NPs).

Silica NPs are very popular and widely used particles in the core shell polymerisation. Due to their inertness, mechanical and chemical stability, and biocompatibility, they have been useful substrates for MIPs intended for sensitive biological applications. In addition, silica possesses many hydroxyl groups, which enables easy polymerisation on its surface. Nevertheless, silica NPs can still be functionalised with vinyl, amino or cyanate groups to ensure better assembly of polymerisation mixture on its surface [91–93].

Other alternatives for core particles are introduced in some papers, which include; titanium dioxide NPs [94], attapulgite resin [95], and polystyrene beads [96].

A very attractive approach for core shell imprinting is the use of magnetite (Fe_3O_4) NPs as core particles. The use of functionalised magnetite particles in the core structure of MIPs imparts a magnetic quality, which can be very beneficial to separate MIPs from complex matrices using a magnet. Recently, this approach has been extensively applied to MIPs in different fields. A search on magnetic MIPs on the MIP database (http://www.mipdatabase.com) in May 2021 gave almost 260 hits, which indicates the high volume of papers concerned to applying this technique in different fields of analysis and extraction [88].

Magnetic MIPs have their widest applications in the field of sample preparation and solid phase extraction (SPE). Their magnetic quality enables their use as a solid support for dispersive solid phase extraction followed by their easy isolation using a magnet [97].

Another equally attractive approach for core shell imprinting is the incorporation of fluorescent NPs in the core of MIPs. Fluorescent functionalised quantum dots (QDs) impart fluorescent characteristics to MIPs that can be easily measured. In the event of target binding, a noticeable fluorescence quenching often occurs, which is detected and used to quantify the concentration of target. Recently, carbon dots (CDs) have been used as an eco-friendlier, less toxic, and more biocompatible option for fluorescent MIPs [5,10,46,98–100]. In addition, silica NPs can also be loaded with fluorescent dyes and incorporated in the core of MIPs [101]. A search on the MIP database (http://www.mipdatabase.com) in May 2021 for fluorescent MIPs gave about 230 hits, which points out to the extensive use of fluorescent MIPs in recent research articles, especially in the field of sensors and biosensors [102].

Fluorescent core shell MIPs should not be confused with fluorescent bulk imprinted MIPs. The later type utilizes florescent monomers or fluorescent dyes such as fluorescein to impart the fluorescent nature. Therefore, the fluorescent element is part of the polymeric material itself and not incorporated in its core [103]. In this type of MIPs, an increase in fluorescence intensity might be noticed in some cases upon binding to the target, which is more analytically accurate than the quenching effect [104].

Some papers combined both fluorescent and magnetic qualities in the structure of the developed MIPs. By incorporating both QDs or CDs and magnetite in the core of MIPs, they could acquire both characteristics for better extraction and detection of target molecules [105,106].

- Surface imprinting after template immobilisation

In this technique, template molecules are immobilised onto a solid support by chemical bonds. Therefore, after polymerisation, the solid support can be removed along with the immobilised template molecules leaving complementary binding sites on the MIPs surface. Thus, the immobilisation of template molecules gives some major advantages; firstly, the immobilisation ensures that the binding sites are all on the surface and well oriented for rebinding of target. Secondly, this technique is useful for insoluble templates, since they do not need to be incorporated in the solvent. Finally, the immobilisation of template via chemical bonds enables complete and easy template removal after polymerisation without the need for excessive washing cycles [107,108].

Micro-contact imprinting is a method that rely on template immobilisation that was introduced in 2005 that is quite simple and effective [109]. Micro-contact imprinting depends on the immobilisation of target molecules on a glass cover slip via covalent bonds. The cover slip is then exposed to the functional monomer to form the prepolymerisation mixture; as to orientate the functional groups of the monomer towards the template molecules in a site-specific manner and create a strong template monomer adduct. Cross linker and initiator are dissolved in the porogenic solvent and spotted as a small drop on a glass slide. The imprinting process then starts by bringing the cover slip in contact with the glass slide and exposing it to UV radiation. After polymerisation, the cover slip is removed and unreacted monomers along with any template molecules that could have leaked into the surface are washed away. Micro-contact imprinting provides an easy way for rapid and simultaneous polymerisation of many templates at the same time. In addition, it is a very useful technique for expensive samples that are only available in scarce amounts, since the procedure requires a minimal amount of template molecules. Due to the previously mentioned advantages, micro-contact imprinting has been successfully used to imprint different target molecules, especially proteins [110–112].

Solid phase imprinting of MIPs is a very innovative and promising technique that depends on template immobilisation as well. In this imprinting method, the template is immobilised via covalent bonds onto the surface of functionalised activated glass beads. The glass beads bearing the template are then brought in contact with the prepolymerisation mixture to initiate the reaction. The polymerisation conditions are set to produce MIPs nanoparticles instead of films; conditions such as high dilution and short polymerisation times are favourable to produce nanoparticles. After polymerisation, the solid support plays another important role, which is acting as an affinity medium (somehow like chromatographic stationary phase). The immobilisation of template on the solid support allows for the removal of unreacted monomers, and low affinity polymer particles from the high affinity nanoMIPs. This is achieved

by washing the glass beads under specific conditions in which the nanoMIPs remain attached while the other unreacted components elute from the system. After successful washing, detachment of nanoMIPs from the glass beads is carried out by washing under a different set of conditions, usually higher temperature and different solvent, after which nanoMIPs are eluted with complementary binding sites and complete template removal [113]. **Figure 4** shows the process of solid phase imprinting and separation of nanoMIPs. Although the yield from this imprinting method is usually small, their binding affinity is much higher than MIPs produced using other imprinting methods. In addition, the yield can be increased by carrying out several cycles of synthesis using the same system, which can be turned into an automated procedure. Solid phase imprinting has its biggest share of applications in the field of protein imprinting and sensor applications [114–117].

3.4.3. Imprinting methods using different polymerisation mechanisms

- Controlled/living radical polymerisation

During free radical polymerisation, there is a high concentration of generated radicals, which results in very fast chain propagation. Consequently, premature chain termination cannot be controlled, which in turn causes the generation of varying length polymer chains. The varying polymer chains are the reason for the broad size distribution of MIPs produced using the free radical mechanism. Moreover, if the monomer has a different radical reactivity than the cross linker, this is will definitely cause a variation in the distribution and orientation of functional groups in MIPs. Because of these reasons, controlled/living radical polymerisation (CRP) technique has been introduced in the field of the molecular imprinting. CRP depends on the use of temporary radical capping agents (dormant species), which aim to protect the radicals from premature termination. By using capping agents, propagation chains are given the time to grow at the same rate and simultaneously until termination, therefore the resulting polymer chains have almost the same size and very narrow size distribution range. One more advantage of CRP is the introduction of living functional groups to the polymer chain end, which can be used to further modify the MIPs structure [118–120].

CRP chemistries like atom transfer radical precipitation polymerisation (ATRP) [121], reversible addition-fragmentation chain transfer (RAFT) [122] and nitroxide mediated polymerisation [123] have all been applied successfully to produce MIPs with very small size and narrow size distribution. However, RAFT is the most versatile and suitable technique due to its applicability to a wide range of monomers, moderate reaction conditions and easy control of the polymer structure via post imprinting functionalisation [124]. Nevertheless, CRP techniques are less utilized in comparison to free radical polymerisation, which is attributed to the fact that CRP requires expensive reagents, and the reactions are usually slow and time consuming.

- Electropolymerisation

Electropolymerisation is a special class of molecular imprinting in which polymerisation occurs on the surface of a conducting electrode such as gold, platinum, glassy carbon electrode and others. Herein, a solution containing the target analyte (template) and suitable functional monomer(s) is subjected to electrochemical energy that initiates polymerisation. As a result, a thin film of MIPs is formed on the surface of the electrode. Therefore, after the removal of template, this film contains gaps or cavities that are complementary to the analyte. Subsequently, upon rebinding to the target analyte in a sample, a signal is generated by the electrode, which is measured by the suitable electrochemical analytical device.

Pyrrole, phenol, scopoletin and o-phenylenediamine are among the most common functional monomers employed in electropolymerisation due to their ease of polymerisation at low potential and their ability to form chemical bonds with a variety of targets. The choice of monomer usually depends on the intended electrochemical analytical method; pyrrole is most commonly used for direct voltammetric assays (potentiometry, square wave voltammetry (SWV), cyclic voltammetry (CV), etc.). However, less conductive monomers, such as o-phenylenediamine are more commonly used in electrochemical impedance spectroscopy (EIS) assays [125].

Electropolymerisation offers a group of advantages that makes it a very appealing technique, especially in the field of sensors. Compared to traditional bulk polymerisation, electropolymerisation is much faster and easier. Moreover, it can be carried out *in situ* on the surface of the working electrode while the whole process is monitored on a computer screen. In addition, the thickness of the produced film is manageable by changing the applied potential, which makes the method more reproducible and accurate. Nonetheless, electropolymerisation also suffers from some of the same drawbacks encountered by other types of polymerisation. Non-specific binding and incomplete template removal are two commonly encountered problems with electropolymerisation, which require lengthy optimisation procedures to overcome and control. However, by overcoming these setbacks, electropolymerisation is a very promising technique whose sensitivity can reach the picomolar levels with ease [125].

3.5. Characterisation of MIPs

3.5.1. Physical characterisation (Morphological)

Physical characterisation provides valuable information about the morphology of the resulting MIPs as well as their size distribution, geometry and porosity. The size of MIPs is a very important parameter, since the smaller the particle size the more surface area available for binding of target. Moreover, as mentioned before, different polymerisation techniques result in different particle sizes, therefore morphological testing can verify the anticipated particle characteristics using a certain polymerisation technique. It is also essential to identify the shape of the produced MIPs, especially if they are intended for chromatographic applications[126].

Electron microscopy (EM) is a very popular technique to determine the shape, geometry and size of MIPs. Scanning electron microscopy and transmission electron microscopy are usually used to compare morphology of MIPs to NIPs or the morphology of MIPs before and after template removal. [126,127].

Other particle size analyzing methods include laser diffraction and dynamic light scattering. These techniques give more accurate data such as mean size, mean surface area and size distribution. However, these methods are less popular than EM, since EM gives a visual approach to the shape of MIPs. In addition, EM can show if aggregates of particles are formed, which can be misinterpreted as large particles while using laser diffraction [127].

To estimate MIPs porosity, two characterisation methods can be used; nitrogen gas adsorption and mercury intrusion porosimetry. These methods provide the ratio between the volume of pores to the total volume of polymer particles. Using these methods, we can investigate the volume and surface area of MIPs pores [127]. One study using nitrogen adsorption revealed that the volume of pores in MIPs is bigger than the ones in NIPs, which confirms that template plays a role in increasing surface area and porosity of MIPs [128].

3.5.2. Chemical characterisation

NMR is a very useful spectroscopic method that is commonly used for chemical characterisation. Both solid state and solution NMR have been used especially in pre-polymerisation studies to study the mechanism of imprinting and determine the bonds formed between the monomer and template [129].

FT-IR is another helpful spectroscopic technique that is very popular in characterisation of MIPs. FT-IR is a handy method regarding functional group analysis; therefore, it is used to ensure complete polymerisation of MIPs. By comparing the spectra of starting monomers and template to the spectra from MIPs, researchers can detect the absence of functional groups involved in polymerisation such as vinyl groups (C=C stretching) in the spectra of MIPs. [130].

UV-visible spectroscopy is by far a method that is widely used by all researchers in chemical characterisation and prepolymerisation studies. UV-vis spectroscopy is an easy and rapid method that can be used to study the bond formed between the template and monomer in pre-polymerisation studies [129]. Furthermore, it is a very successful tool for ensuring complete washing of MIPs by analyzing batches of washing solution for presence of template. In addition, UV-vis spectroscopy is the main method used in rebinding experiments to detect and quantify the template or competitor molecules in supernatant solutions [131].

3.5.3. Thermal characterisation

Methods like thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) have been applied in many research projects on MIPs. TGA is suitable to determine the presence of residual solvent molecules or unreacted monomers or cross linkers. In addition, it gives an insight about the thermal stability of MIPs and the temperature range at which they can be used with no stability issues. TGA is also useful to provide a proof of imprinting, since it is anticipated for MIPs to have increased thermal stability in comparison to NIPs [129,132,133]. On the other hand, DSC is a method that exposes materials to different cycles of heating and cooling, this in turn provides information about the crystallinity of the polymers. This can be of interest if MIPs are intended to be immobilised or deposited on solid support as in the case of sensors or biosensors. However, there is a scientific debate if DSC can be used to compare MIPs to NIPs; some papers show there is a noticeable difference in DSC patterns yet some other studies do not [127].

3.5.4. Functional characterisation

Functional characterisation or in another expression binding tests are several experiments carried out to investigate the binding properties of the developed MIPs. These tests aim to measure binding to the target in quantifiable factors through certain mathematical formulas. Moreover, binding to competitive analytes that are similar in structure to the target or that can coexist with it in the same matrix is estimated. Analytical methods like UV-vis spectrometry or LC-MS are used to monitor the outcome of binding experiments [131].

a) Binding affinity

In order to calculate the binding affinity, we carry out a rebinding experiment, in which we incubate a certain weight of MIPs or NIPs with specific volume of target solution for a certain period of time until equilibrium is reached. Analytical methods such as UV spectrometry is

(eq. 1)

used to determine the remaining concentration in the solution after binding. The following equation is used to calculate the binding affinity for both MIPs and NIPs:

$$Q = \frac{C0 - Ct}{m} \,.v$$

Where, Q is the adsorption amount, C_0 is the initial concentration, C_t is the remaining concentration after equilibrium, m is the weight of the polymer used in the rebinding experiment, and v is the volume of the target solution added. Note that v and m should be the same in both experiments for MIPs and NIPs.

The higher the value of Q the greater the binding affinity. However, the value of Q for MIPs should always be higher than that of NIPs, due to the selectivity of MIPs for the target compared to the nonspecific binding of NIPs.

b) Binding selectivity

MIPs tend to have different non-specific binding sites (due to reasons that will be discussed further). Therefore, it is essential to estimate the selectivity of the developed MIPs to the target analyte to ensure that non-specific binding is not a problem.

One method to study binding selectivity is to compare the binding of MIPs against the binding of NIPs. This comparison confirms the presence of specific binding sites in MIPs for the target that are absent in NIPs. Imprinting factor (IF) is a calculated parameter that is defined as the ratio between the adsorption amount (Q) of MIPs to that of NIPs for a particular analyte under a specified set of conditions.

$$IF = \frac{Q_{MIPs}}{Q_{NIPs}} \qquad \text{(eq. 2)}$$

From equation 2, we deduce that the IF value should be higher than 1; the higher the value of IF the greater the difference between MIPs and NIPs, which in turn proves the presence of selective binding sites in MIPs.

However, comparison to NIPs may not be the best way to ensure selectivity of MIPs. This can be attributed to two main reasons. Firstly, the structure of NIPs tends to be more compact due to the absence of template during polymerisation, therefore the number of available free binding functional groups is by nature less than MIPs. Secondly, a published study has shown that during optimisation of MIPs synthesis, the optimum composition of MIPs that has the highest binding capacity corresponds to NIPs that bind the target strongly as well [134].

A better way to estimate selectivity is to compare binding of target to MIPs with binding to a competitive analyte under identical experimental conditions.

Herein, we calculate a factor called selectivity factor (α) for every competitor analyte we test in binding experiments.

$$\alpha = \frac{Q_{MIP.target}}{Q_{MIP.competitor}} \qquad \text{(eq. 3)}$$

The selectivity factor (α) should have a value higher than 1; the high values of α for different tested competitors are numerical proof of selectivity of MIPs.

c) Binding kinetics

When discussing binding kinetics, we must make an important note that binding/release of target to MIPs is a diffusion limited process. In other words, molecules of target need to undergo mass transfer from solution phase to solid phase of MIPs. This can be a very slow process, since molecules need to cross the porous solid polymeric network to access the binding sites. In the case of surface imprinting, binding is usually faster, since binding sites are on the surface and readily accessible [135].

The best way to calculate binding kinetics is to carry out a batch rebinding assay for both MIPs and NIPs. A batch rebinding experiment is conducted by incubating the exact same mass of MIPs or NIPs in the exact same concentration of target analyte solution, and then analyzing aliquots of the solution over different time intervals. The graphical relationship between time and the free concentration (F) in mol. L^{-1} can be used to determine equilibrium time and if binding kinetics follow first, second or third orders.

On the other hand, binding isotherm is another experiment in which the exact same mass of MIPs or NIPs is incubated with different concentrations of target analyte. After equilibrium is reached, aliquots of the supernatant solution are analysed to determine the concentration of free target (F) in mol. L^{-1} and determine bound concentration (B) in mol. L^{-1} using equation 4.

$$B = \frac{ntarget}{m} - \frac{v}{m} x\ F \qquad \text{(eq. 4)}$$

Where n_{target} is the total number of target mols used in the experiment. The binding isotherm is a graphical relationship between B and F. Isotherm for MIPs is usually not linear; it reaches a plateau at high F values indicating the saturation of the binding sites. However, the curve for NIPs is usually linear and indicates non-specific binding due to the randomly oriented functional groups on its surface. The difference between MIPs' and NIPs' curves is attributed to the specific binding of MIPs due to the presence of selective binding sites. Therefore, optimisation is carried out to maximize the difference between the two isotherms, which is reflected on the selectivity of MIPs.

Note that the word "isotherm" means constant temperature. Therefore, binding kinetics experiments should be carried out under constant temperature, as well as constant solution conditions (pH, ionic strength, solvent, etc.) for all concentrations of the tested target.

Data from binding isotherms can be further fitted into theoretical models, such as Langmuir model or Langmuir-Freundlich model. The curve which fits the experimental data best can give us an estimate of the number of binding sites and their binding affinities [136].

3.5.5. Characterisation of MIPs films

For MIPs imprinted as thin films or on the surface of a chip, a crystal or an electrode to be used as sensors, other characterisation methods may be applied for immobilised MIPs. Atomic force microscopy, X-ray photoelectron spectroscopy, quartz crystal microbalance analysis (QCM), and surface plasmon resonance (SPR) are all useful techniques that can be used alone or in combination with EM to characterize on-surface MIPs. Such methods give us valuable information regarding surface homogeneity, roughness, and to ensure complete deposition of MIPs on the solid support. In addition, they can give an online monitoring of binding events as well as to ensure complete template removal [127].

3.6. Advantages, challenges and drawbacks in MIPs synthesis

Among the most important merits provided by MIPs are their stability, which enables their use under different sets of non-physiological conditions. Moreover, they can be stored for a long time at ambient temperature with no damage, degradation or loss of target recognition efficiency. In addition, the process of synthesis of MIPs is a far more cost effective and time saving procedure compared to production of antibodies. These advantages are very economically appealing traits, especially when MIPs are designed to be produced on an industrial scale. Furthermore, as we previously mentioned, MIPs can be easily functionalised with fluorescent or magnetic labels, they can also be manufactured as films, particles or nanotubes, therefore they can be tailored according to the intended use to maximize their potential, which is something that cannot be achieved with antibodies [137]. A recent study by K. Smolinska-Kempisty et al. has compared the performance of nanoMIPs against antibodies used in an ELISA assay to a specific target. The results proved that nanoMIPs are equally effective or even better than antibodies, which makes them better candidates for industrial diagnostic applications [138].

However, despite the many high value qualities offered by MIPs, it is still a field of research that has many challenges to overcome. MIPs synthesis is a tricky field with too many variables, and without control over these variables MIPs might not be useful for their intended purpose or even worse to cause a false result. Moreover, MIPs synthesis is mainly dependent on the chosen template, some templates are relatively easy to imprint yet others present their own set of difficulties. Herein, we discuss the most common challenges encountered in MIPs synthesis with the most recent proposed solutions.

3.6.1. Binding site heterogeneity

Binding site heterogeneity is considered an inevitable setback when developing MIPs. This problem usually results in MIPs that are "polyclonal" in nature, thus they might exhibit non-specific binding with other species in the target sample. Binding site heterogeneity is one of the main problems that limits the wide applications of MIPs and gives antibodies a significant leverage on MIPs, since antibodies are "monoclonal" and highly specific.

The drawback of binding site heterogeneity is caused by different factors and in almost all types of imprinting, yet it is more prominent in non-covalent imprinting. One of the obvious reasons for this problem is that the monomer-template interaction is a process governed by equilibrium. Therefore, during equilibrium, prepolymerisation mixture in fact contains too many species of template-monomer interactions, different complex ratios, not just 1:1, in addition to free monomers. Furthermore, some side interactions occur between the template and cross linker, or between the monomer and the cross linker or even between template molecules themselves forming clusters. These chaotic species occurring in the prepolymerisation mixture are translated into the final polymer, forming many non-specific binding sites. However, another array of factors also contributes to binding site heterogeneity after polymerisation, this includes grinding and sieving of polymer, template removal process, and using different solvents during rebinding experiments. All these factors cause further heterogeneity by damaging the binding sites, collapsing sites after template removal, and variable swelling/collapsing of the polymer upon exchange of the solvent [139]. **Figure 5** is a cartoon representation of how heterogenic binding sites can be generated during polymerisation and after.

Semi-covalent imprinting is considered a very promising imprinting method to reduce binding site heterogeneity to the minimum. Since this method combines the merit of covalent binding (control on the template-monomer interaction) and the merit of non-covalent release

(reduced kinetic restriction), the method offers better control of the functional groups orientation on the binding sites and therefore reduces the resultant heterogeneity [39,40].

Stoichiometric non-covalent imprinting is another useful approach to reduce binding site heterogeneity. This approach was introduced first by Wulff et al. [140] and depends on reaching a high association constant between template and monomer, this is achieved by choosing a functional monomer that is capable of forming two or more hydrogen bonds with the template. Using this method, we combine the high splitting yields and rapid binding kinetics of non-stoichiometric, non-covalent imprinting with the precise control over functional groups orientation and good accessibility to binding sites of typical covalent imprinting [141].

Despite the use of the prior alternatives, semi-covalent, stoichiometric non-covalent or even covalent imprinting still suffer from some binding site heterogeneity. This is attributed to the difference in the outer sphere structure in the final polymer; this essentially means a difference in the binding sites accessibility. Moreover, these methods do not eliminate the heterogeneity arising from grinding, sieving and the collapse of binding sites on template removal and solvent exchange [142].

Surface imprinting is a more convenient method to reduce the binding site accessibility problem. Since the binding sites are all oriented on the surface, this can reduce the difference in the outer sphere nature of the binding sites [142].

3.6.2. Template bleeding

As mentioned before, after successful molecular imprinting, template removal should be attempted using a suitable solvent or reagent. In the case of inefficient template removal, some of the template molecules remain stuck to MIPs' binding sites. Under these circumstances, the entrapped template molecules can be released later during the application of MIPs to their intended purpose in a phenomenon known as template bleeding or "template leakage". Template bleeding is another problem that significantly hinders the applications of MIPs in the field of sensors and chromatographic separations. This is because if the attached template molecules are released during the sensing process, a false positive result can be obtained. Moreover, in the case of chromatographic separations, template bleeding can cause a poor chromatographic separation and possible contamination of the fractionated sample components [139,143].

There are some proposed solutions for the problem of template bleeding including isotope molecular imprinting, microwave assisted extraction or carrying out a blank extraction [144]. However, among the best and most widely used solutions for the template bleeding problem is dummy imprinting.

Dummy imprinting is a process that refers to the imprinting of a compound that is structurally similar to the target compound instead of the target itself. There are two types of dummy imprinting: firstly, fragment imprinting, which utilizes a part of the target molecules as the dummy [145]. Secondly, interval immobilisation imprinting, which utilizes an entirely different compound than the target, however this compound has the same distance between two identical functional groups to the target compound [146]. Using either of the dummy imprinting approaches, if template leakage occurs, it will have no significant effect on the result or the accuracy of the testing method. In addition, dummy imprinting is a very suitable alternative if the template is expensive, dangerous or unstable. Nevertheless, dummy imprinting is not always a viable solution, since finding suitable dummies is not always simple.

Combining solid phase imprinting with a dummy imprinting technique can introduce an excellent solution for the problem of template bleeding. However, the template extraction process can still be slow and some researchers presume complete template removal in the case of proteins is almost impossible [147].

3.6.3. Imprinting of hydrophilic templates and application to aqueous media

Hydrophilic and polar compounds present a challenge when they are used as templates for MIPs, since they are practically insoluble in organic solvents. Across the years, researchers came up with few effective solutions for the problem of hydrophilic template imprinting. Among the first proposed solutions is the structural modification of the template molecule to introduce a hydrophobic alkyl chain; using this approach the solubility of the template in organic solvent is expected to increase [148]. However, the process of structural modification is time consuming and may alter the functional groups needed for imprinting. Another approach is the use of a hydrophobic dummy as an alternative for the original target, yet finding a suitable dummy is not always easy. Up till now, the simplest and the most effective approach for imprinting hydrophilic templates is using ion-pairs. This technique relies on the formation of ion pair between the hydrophilic template and a surfactant. Following the formation of the ion pair, it is extracted by liquid-liquid extraction (LLE) using organic solvent and molecular imprinting proceeds as normal [149].

Solving the problem of imprinting of polar templates does not necessarily mean that the developed MIPs can be applied in aqueous media. MIPs developed in organic solvents show weak recognition to the target in water or polar samples due to the disrupted hydrogen bonds. Micro liquid-liquid extraction is a useful way to extract the template molecules from their aqueous samples using minimum amount of organic solvent and then the extracted target is loaded onto the solid MIPs [150]. Another reasonable approach is to use hydrophilic monomers for the synthesis of MIPs for polar templates. Hydrophilic monomers such as (hydroxyethyl)methacrylate (HEMA) or β-cyclodextrins can be used in water as a porogenic solvent to synthesise MIPs that are compatible with aqueous media [151,152].

Hydrophilic properties can be imparted on the developed MIPs through the process of surface modification. R. Song et al. prepared hydrophilic MIPs through surface modification of the imprinted microspheres with an ultrathin hydrophilic shell made of charged monomer (MAA) and uncharged monomer (poly(NIPAm)) for the detection of glutathione in aqueous media [153]. Surface modification of MIPs is a very effective and relatively easy method to impose a hydrophilic nature to the developed MIPs and in turn increases their applicability.

Recently, there have been a trend to utilize green and natural polymeric materials in the synthesis of MIPs. In the light of this trend, researchers have adopted the use of natural polymers or their derivatives such as polysaccharides or gelatin. Polymers made using natural materials show great applications in aqueous media due to their hydrophilic nature. Moreover, due to their natural origin, these polymers exhibit amazing properties such as low toxicity, biocompatibility, low cost and biodegradability [154]. L. Xu et al. prepared two hydrophilic MIPs for alpha-Lipoic acid through the use of chitosan as functional monomer cross linked once with epichlorohydrin and another with glutaraldehyde. Both MIPs showed great binding selectivity and good applicability in aqueous media [155]. In addition, X. Zheng et al. prepared MIPs membranes for L-tyrosin via the use of gelatin and chitosan as monomers in polyethylene glycol as a porogenic solvent. The resultant MIPs membranes showed high selectivity and permeability for targets and were suitable for use in aqueous media [156].

A special class of MIPs, which are prepared using a metal coordination bond between template and the functional monomer can be effectively used in polar solvents; owing to the fact that metal coordination is a strong bond that is not easily disrupted by water molecules unlike hydrogen bonds. 8-hydroxy-2'-deoxyguanosine MIPs were prepared utilizing metal coordination bond. Methacryloylamidohistidine-platinum(II) was used as the metal chelating monomer and therefore rebinding of template depended on formation of coordinate bonds. Consequently, the developed MIPs were incorporated in a sensor to detect 8-hydroxy-2'-deoxyguanosine in aqueous samples [157].

3.6.4. Protein imprinting

The challenges posed by proteins as targets exceed the normal challenges encountered with other hydrophilic templates. The large molecular weight of proteins ranging from a few Daltons to thousands of kilo Daltons is considered a big problem in protein imprinting; The large molecular weight of proteins makes it difficult for protein molecules to diffuse through the highly cross-linked polymeric structure, this results in very slow binding kinetics. Moreover, the large molecular weight may also impede the efficient template removal after MIPs synthesis causing the phenomenon of template bleeding or reducing binding efficiency. Furthermore, proteins have poor solubility in organic solvents, which are commonly used in molecular imprinting and they are by nature sensitive to the extreme conditions required for radical polymerisation, such as elevated temperatures and different pHs. Therefore, protein imprinting needs to be carried out in conditions, which are similar to their natural physiological conditions, not only to prevent their degradation but also to maintain their conformational integrity [139]. In addition to the aforementioned difficulties, protein molecules themselves have different recognition sites on their surface including charged amino acids and hydrophilic or hydrophobic regions, that can increase the chance of MIPs reduced selectivity and cross reactivity with any other protein molecules containing similar regions or charges [158].

Bulk polymerisation is considered the least popular choice for protein imprinting. This is attributed to the fact that proteins in solution have multiple conformational variations that can result in a very broad range of binding sites and non-selective binding. Moreover, the use of polar porogenic solvents favourable for proteins can reduce the strength of hydrogen bonds between the template and monomer resulting in reduced binding efficiency [143].

In order to solve the problems associated with protein imprinting, epitope approach has been introduced. In 2001, Rachkov and Minoura [159] introduced the epitope approach as a new way for protein imprinting. In this approach, a small peptide segment is driven from the big structure of the template protein (usually the C-terminal sequence) and used in the imprinting procedure. In this way, the produced MIPs will be able to recognize this sequence in the whole protein molecule with higher degree of selectivity and minimal non-specific binding. A sequence of no less than 9 amino acids is chosen from the C-terminal of a template protein. The reason for choosing the C-terminal is that it is less prone to post-translational modifications, which increases the chance of its recognition in sample by the developed MIPs. The epitope approach is an attractive alternative for imprinting of bulky protein templates (such as immunoglobulins) that provides the advantages of selectivity, high affinity and sensitivity. Moreover, this method is very cost effective, since it avoids the use of highly pure authentic proteins that might be very expensive. Nevertheless, finding the suitable epitope for protein imprinting is a complex procedure and requires knowledge of protein conformations to predict the exact structure of the produced peptide segment [160–162]. Another recent

approach in epitope imprinting is the use of hidden or internal epitopes. This approach was introduced first by Bossi et al. in which the protein template is exposed to enzymatic digestion or heat denaturation to expose the hidden peptide sequences in its structure. Those epitopes are usually hidden inside the folded structure of the protein and less exposed to the surface interactions. Imprinting on the hidden epitopes increase the recognition of MIPs immensely since after denaturation these peptide sequences become exposed and specifically bind with MIPs without interference from steric hindrance. The choice of the peptide sequence is usually an *in-silico* procedure and requires careful study of the protein structure [163].

As mentioned before, a metal coordination bond is an effective way for molecular imprinting in aqueous media since the metal coordination bond in much stronger than the hydrogen bond and less affected by water. Moreover, relying on the metal coordination bond ensures highly specific binding to the target protein and efficient binding in aqueous matrices as blood. However, the use of metal coordination is limited to protein targets that contain exposed histidine residues on the surface, which are capable of forming such bonds [157,164].

Hydrogel MIPs are a class of MIPs prepared using hydrophilic monomers and low concentration of hydrophilic cross linkers such as polyethylene glycol. The resulting MIPs are in a gel form and have relatively small cross-linked structure. Hence, hydrogels are considered a good alternative for protein imprinting, since the template can easily diffuse through the low cross-linked mass to their specific binding sites due to the high permeability [165,166].

Finally, a novel method for protein imprinting is to combine the template immobilisation technique with the use of a sacrificial support. Template proteins are immobilised on the surface of nano structured polymeric material such as alumina membranes, nanotubes, nanorods or nanofilaments. This method combines the advantages of template immobilisation with the huge surface area provided through the use of nano materials. The large surface area enables easy and rapid access of template protein molecules to their target sites and therefore, provides rapid detectability and response that can be beneficial for the application to sensors [167–169].

3.7. Applications of molecularly imprinted polymers

3.7.1. Drug delivery

Some researchers consider MIPs to be the future for a very selective and controlled drug delivery. The usual polymeric drug delivery systems suffer from some drawbacks the most famous of which is the bursting and sudden release of the drug due to the harsh surrounding conditions. MIPs can provide an alternative carrier system that excels the mundane polymeric materials in many aspects. Because of the high affinity of the designed MIPs to their target drug, they are able to provide sustained and slow drug release, which is highly desirable in the field of pharmacotherapy. Moreover, MIPs offer high drug loading capacity, increased stability against various harsh conditions, handy control over their cross linked structure and the ability to introduce magnetic or fluorescent labels to facilitate *in vitro* or *in vivo* testing and imaging [170].

To date, MIPs have been Introduced in many pharmaceutical forms to overcome the problem of low bioavailability or to provide controlled drug release. Soft contact lenses made of MIPs hydrogel were designed and introduced as an ophthalmic route of administration for glaucoma medications [171], as well as their applications in dermal and transdermal delivery

of drugs such as propranolol [172]. Furthermore, magnetic nanoMIPs were introduced as a smart intravenous route of administration of the anticancer drug 5-fluorouracil for breast cancer patients [173]. MIPs also come in handy in the oral route of administration, since their high stability can prove effective against the different pHs encountered in the gastrointestinal tract. For instance, MIPs were developed for the oral administration of the drug doxorubicin through the use of the biocompatible chitosan monomer [174].

However, note that the application of MIPs as drug delivery system has some hurdles, such as the toxicity and biocompatibility issues with some monomeric materials, ensuring reproducible industrial scale synthesis, and the need for complete pharmacokinetic and pharmacodynamics profiles study. However, these setbacks should be easily overcome by the continuous development and research [175].

3.7.2. MIPs as catalysts

If a person looks closer, they will be able to find an uncanny resemblance between the structure of MIPs and natural enzymes. It was that similarity that led researchers to investigate the potential use of MIPs as catalysts in different chemical or biochemical reactions. Catalytic MIPs can be imprinted on template analogues of the transition state of a specific reaction. Consequently, when these MIPs are introduced into the reaction, they can selectively bind to the transition state and stabilize it, and therefore decrease the activation energy required for the reaction. Alternatively, MIPs can be designed to selectively bind to the reactants or the products to be able to steer the reaction forwards or backwards. Another approach is to develop MIPs as selective carriers for metal NPs required to catalyse any reaction and that in turn facilitates their easy removal after completion [176].Collectively, the recent advances and applications of MIPs in the field of catalysis are illustrated in the review by S. Muratsugu et al. [177].

3.7.3. MIPs in chromatography and sample pretreatment

In the field of analytical chemistry, MIPs provide the greatest contribution either in the fields of separation or sample extraction. The high selectivity of MIPs in addition to their previously mentioned positive traits make them excellent candidates for their use as stationary phases in different chromatographic methods. The combination of the high affinity of MIPs with the advanced technology of high-performance liquid chromatography (HPLC) was a leap forward in the field of separation science. Different shapes of MIPs including NPs, monoliths and spherical beads have been utilized as packing materials for columns used for HPLC separations. H. Xiao et al. prepared surface imprinted MIPs on the surface of silica microspheres using myricetin flavonoid as a template. The prepared MIPs were packed in a stainless-steel column and used for HPLC separation of myricetin from a mixture containing three other flavonoids with high efficiency and selectivity [178].

MIPs come in handy as well in racemic separations using HPLC. Through tailoring and optimisation of MIPs synthesis, scientists could create MIPs that are capable of separating different enantiomers of the same compound and apply them for HPLC separations. Enantioselective separation of phenylalanine enantiomers was achieved using MIPs developed via bulk polymerisation for the separation of L-phenylalanine in its racemic mixture [179]. Another attempt for successful separation of racemic antihistaminic drugs using enantioselective MIPs beads was developed by suspension polymerisation method in aqueous media. Through the high selectivity of the developed MIPs, two antihistaminic drugs: d-chlorpheniramine and d-brompheniramine were separated from their racemates. This is

particularly important in the field of medicine due to the drastic difference in medical efficacy between the two enantiomers of the same drug. More insights into the applications of MIPs in the field of chiral separations were introduced in some useful reviews [180,181]. The only precaution that we like to highlight regarding the application of MIPs in chromatography is the peak tailing that results from binding site heterogeneity. Therefore, care should be taken during synthesis and optimisation of MIPs intended for chromatographic use to avoid this problem.

Sample extraction and purification is one of the most important steps required in almost all analytical procedures. SPE is a very common extraction technique used for such complex samples. It provides various advantages, some of which are high efficiency, rapid separation, reproducibility, and readiness for automation, especially in comparison with other extraction methods such as LLE. The application of MIPs as stationary phases inside the SPE cartridge added even more appealing qualities to SPE. The stability of MIPs made SPE suitable for samples with extreme pH values or those samples separated at elevated temperatures. In addition, MIPs are highly effective in the presence of organic solvents usually used to elute the samples. Furthermore, the cost effectiveness and easy procedure of MIPs synthesis can contribute to reducing the cost of SPE cartridges [182].

An extensive literature search revealed over 350 publications utilizing MIPs into the field of SPE, which only emphasizes the great role played by MIPs in the field of analytes' extraction. Regarding medical applications, one of many examples includes application of a MIPs SPE cartridge to extract and detect cotinine alkaloid. Cotinine can be used as a biomarker of nicotine exposure in both active and passive smokers. Researchers in this work were able to develop a SPE cartridge specific for cotinine that was easy to use, quick and reusable to extract cotinine from saliva of smokers [183]. MIPs SPE is also very helpful in the field of food analysis; A group of researchers were able to combine a core shell imprinting technique with a dummy imprinting approach to create magnetic MIPs SPE specific for aminopyralid herbicide. Picloram was used as a dummy and molecular imprinting was carried out on the surface of carboxyl and amino functionalised magnetite particles. The developed MIPs SPE cartridge was able to separate and detect aminopyralid in milk samples to be further analysed by LC-MS. In the scope of forensic analysis, MIPs also left a fingerprint. T. Murakami et al. developed MIPs SPE system for extraction of the addictive cathinones from blood and urine samples. The recovery of drugs using MIPs SPE was found to be higher than the conventional SPE and LLE, which is a solid proof for their efficacy in this field [184].

The offline SPE approach used in the aforementioned examples is relatively easy and flexible, however, it consumes significant amount of solvent, and samples are prone to contamination prior to being analysed, therefore the online SPE systems are more favourable. In online approach, analytes separated with SPE are automatically introduced into an analytical instrument such as HPLC or GC for further quantitative analysis. A recent example is introduced for the extraction and quantitation of the mycotoxin patulin. Herein, researchers developed surface imprinted MIPs for the extraction and preconcentration of trace amounts of patulin in fruit derivatives via SPE. Following the extraction, samples were automatically loaded into HPLC column for analysis. The online approach here was very useful, since the concentration of target was very low. Moreover, no possible contamination of the analyte could have happened, since the samples were directly injected into the HPLC system with no need for an operator [185].

Collectively, MIPs find their widest applications in the area of analysis, however they are a more popular choice for sample pretreatment to extract the target analytes prior to their quantitation.

3.7.4. MIPs in the field of sensors and biosensors

A sensor is defined as a device that measures a specific chemical reaction and produces a certain signal that is corresponding to the concentration of the analyte involved in this reaction. In case the analyte is a biological molecule, the device is called a biosensor. There are three main elements in the structure of any sensor namely, the analyte, which is the target molecule that needs to be detected or quantified, the bioreceptor, which is responsible for selective binding of the target molecules in the sample and production of a signal in different possible forms (light, pH change, or charge change, etc.). Finally, there is the transducer, which is responsible for converting the resultant signal from the binding event into a measurable electrical signal proportional to the concentration of target and can be displayed as readable units [186].

From this brief explanation about the nature of sensors and their structure, we see clearly where MIPs can fit in. For the past decades, natural molecules such as antibodies or enzymes have been used as the bioreceptor as in the case with ELISA. However, due to the great advantages offered by MIPs over natural antibodies, MIPs are now leading the race in the field of sensing and biosensing.

The first type of MIPs that can come handy in the field of sensing is fluorescent MIPs. Utilizing this type of MIPs in a sensor device can translate the change in fluorescence that happens upon binding of the target to give a signal proportional to the analyte concentration. T. Zhou et al. developed a fluorescent nanosensor for the detection and quantitation of the antibiotic tetracycline. Researchers prepared fluorescent core shell imprinted polymers on the surface of graphene QDs. The binding of these MIPs to tetracycline caused fluorescence quenching, which was proportional to tetracycline concentration. Moreover, the authors applied the developed sensor to measure tetracycline in real milk samples and recovery percentages were promising, which in turn points out the great potential for MIPs in this field [187]. The same sensor principle was also applied to larger targets including proteins such as lysozyme and the produced sensor revealed high efficacy and applicability in complex biological samples [188]. Most of the recent applications of fluorescent MIPs in the field of sensing have been summarised in a review [189].

Electrochemical sensing is also a highly explored type of sensors in which the binding event translates into an electrochemical signal. MIPs tagged with a redox probe are utilized in electrochemical sensing of different analytes. The selective binding of target causes conformational changes in the structure of MIPs, which are transformed into an electric current, thereby the concentration of target can be measured via different electrochemical methods [190]. Nevertheless, the efficiency and accuracy of electrochemical MIPs based sensors are sometimes limited by the weak electrochemical conductivity of the cross-linked structure of MIPs. Consequently, Y. Li and his group prepared a smart type of MIPs imprinted on magnetite nanobeads and gold NPs to be deposited on reduced graphene oxide electrode. The combination of reduced graphene and metal nano particles was a brilliant strategy to boost the response current and increase sensor efficiency. The developed sensor was used to detect ractopamine analyte in water samples with very high sensitivity using CV and EIS techniques [191]. Progress and perspectives of MIPs in electrochemical sensing are discussed in detail in a recent review [192].

Capacitive sensors are a type of affinity sensors, which measures the change in dielectric properties (capacitance) upon binding of the target. N. V. Beloglazovaa et al. prepared a capacitive biosensor combined with MIPs for benzo(a)pyrene detection in water samples. MIPs were prepared using two different polymerisation techniques, the first produced MIPs beads, which were covalently coupled to the electrode, the other method involved *in situ* synthesis and deposition of MIPs on the electrode surface. The performance of the two types of MIPs as bioreceptors was compared to each other and to natural antibodies. The results of the study showed that the latter approach of MIPs synthesis provided better results compared to MIPs beads. In addition, sensors prepared using antibodies had a wider linear range, yet MIPs sensors showed higher stability and reusability [193]. Another capacitive biosensor was prepared by electropolymerisation of the naturally derived compound resorcinol to prepare polyresorcinol MIPs for the antibiotic sulphanilamide. The prepared sensor was applied to detect and quantify sulphanilamide in milk, tap water and drinking water with no need for preconcentration steps [194].

A state-of-the-art technique for sensing that is increasingly applied nowadays is SPR. SPR is a type of optical sensor that depends on measuring the angle of reflection of a polarized light beam off an electrode surface separating two media. Usually, bioreceptors are immobilised on the other side of the electrode, which upon binding of the target analyte produces a change in the angle of reflection that can be directly correlated to the concentration of the analyte. Combining MIPs with a very sensitive technique such as SPR is very valuable in the field of analytical chemistry and microanalysis. A MIPs SPR biosensor was prepared for detection and quantification of iron regulating hormone hepcidin. The sensitivity of the biosensor reached a remarkable picomolar range, which is very useful in testing real samples [195]. Another SPR MIPs based sensor was developed for the analysis of amoxicillin antibiotic. The developed MIPs were coupled to two sensing techniques, SPR and QCM. Although the SPR method showed a higher limit of quantitation, both methods were effectively applied to detect and quantify amoxicillin in real egg samples [196]. This cited review discusses further data on MIPs coupled to SPR and other optical techniques in the field of biosensing [197].

Finally, acoustic wave sensor is a type of mechanical sensor, which utilizes a piezoelectric material such as quartz to generate the acoustic wave by alternating current induced oscillation. These sensors are sensitive to mass changes occurring on its surface as well as the change in the physicochemical environment. Coupling of MIPs to surface acoustic sensors is very advantageous in the sensing field, especially after the recent application of acoustic sensors in liquid media. MIPs deposited as films on acoustic sensors such as QCM [198] or the higher frequency and higher sensitivity LOVE wave sensor have created a very useful and sensitive class of sensors for different target analytes [199].

Eventually, the future for MIPs' applications is coupling it to cutting edge sensing technologies. Sensors are needed now more than ever to provide rapid, sensitive and onsite detection of different chemicals and biomolecules. Miniaturized sensors provide the basis for lab on chip or portable reactors that can be used anywhere to provide reliable results in the fields of medicine, forensics, food and environmental analysis.

4. Critical review concerning the application of MIPs in the field of protein biomarkers' analysis

Over the past ten years a large volume of papers has been published that utilizes the technology of MIPs in the field of biomarkers' analysis in general. The previously mentioned merits of MIPs have made them a very attractive approach for detection, extraction, enrichment and sensing of different analytes more specifically biomarkers. Being the predominant types of biomarkers, proteins biomarkers have been chosen as the focus of this critical review. Moreover, due to the aforementioned difficulties in imprinting of proteins, we want to highlight how these difficulties have been addressed and treated. We like to point out that this review is aimed to help future researchers to concentrate on more critical points in MIPs research, gain further insights on the orientation of the scientific community in the MIPs' field, understand the common setbacks encountered in molecular imprinting of proteins, and finally to try finding possible alternatives or solutions for some of the common issues.

Through a literature survey covering the past ten years, we have found numerous scientific papers describing the application of MIPs on protein biomarkers. The reviewed papers were classified according to the diseases signaled by biomarkers in order to provide a base for comparison. **Table 1** summarises all the collected papers in the field, highlighting all important aspects such as MIPs composition, technique of polymerisation, limit of detection (LOD) and applications.

From **Table 1**, it is apparent that the largest volume of published papers is concerned with cancer biomarkers. Almost half of the total paper count is related to different cancer biomarkers. This is attributed to the highest priority of cancer research and the continued efforts to find simple, reliable and fast methods for early detection of this disease. Nonetheless, we can also find significant applications to other disease's biomarkers such as cardiac diseases, metabolic disorders, and neurological diseases.

Another significant point we can see from **Table 1** is that the majority of applications of MIPs are in the sensing field, especially electrochemical sensing. This indicates the successful pairing of molecular imprinting technology with sensing technology, which is expected to be a superior alternative to common sensing and diagnostic methods such as ELISA. Furthermore, the increased electrochemical sensing methods in all the recent papers can be due to the high sensitivity of electrochemical analytical methods, which when combined with the high selectivity of MIPs can reach very high sensitivity levels. As observed, LOD can reach picogram or picomolar levels with ease when using electrochemical sensing methods.

Finally, we can find that most researchers were very eager to apply their developed methods to biological fluids. This is of prime importance if the developed method is to be taken further and be used as an effective diagnostic tool for any of the aforementioned diseases. Some of the applications are performed on real serum or urine samples from actual patients, however the majority are performed on spiked samples.

Table1: MIPs based analytical methods and sensors applied to protein biomarkers for different diseases.

Biomarker	Technique of polymerisation	Composition of MIPs Monomer, cross linker, initiator, porogenic solvent	LOD	Applications	Year and reference
Prostate cancer biomarker					
PSA	Template immobilisation on graphene oxide beads.	Vinyl benzoate & (vinylbenzyl)trimethylammonium chloride & AA, MBA, BPO, Hepes buffer.	0.0583 nM	Potentiometric sensor applied to spiked artificial serum samples. Researchers applied the same sensor to cell culture samples in another latter paper.	2014 2016 [200,201]
PSA	Template immobilisation with DNA aptamer on a gold electrode followed by electropolymerisation.	Dopamine.	1 pg mL^{-1}	Electrochemical impedance detection and quantitation of PSA without application to biological samples.	2016 [202]
PSA	Precipitation polymerisation via RAFT.	AA & 2-(dimethylamino) ethyl methacrylate, EGDMA, Macro-CTA poly hydroxyethyl methacrylate and normal cumyl dithiobenzoate (RAFT reagents), MeOH: water (4:1 v/v).	0.013 ng mL^{-1}	LOVE wave acoustic sensor applied to spiked mouse serum.	2018 [203]
PSA	ATRP followed by post imprinting capping.	4-[2-(N-methacrylamido) ethylaminomethyl]benzoic & 2-methacryloyloxyethylphosphorylcholine, MBA, ascorbic acid, phosphate buffer (pH 7.4).	5.4 ng mL^{-1}	SPR sensor without application to biological samples.	2019 [204]
PSA	Electropolymerisation on gold screen-printed electrodes.	Pyrrole and initiation was done via CV in potassium chloride solution.	2 pg mL^{-1}	Electrochemical nanosensor applied to	2019 [205]

				real serum samples from patients.	
PSA & Myo	Surface imprinting on screen-printed electrode.	Am, MBA, APS, 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide ester).	5.4 pg mL^{-1} 0.83 ng mL^{-1} for PSA and Myo.	Dual template EIS sensor combined with an immunosensor for PSA only with application to real serum and urine samples.	2019 [206]
PSA	Electropolymerisation on gold electrodes.	Pyrrole and initiation was done via chronoamperometry in PBS buffer (pH 7.4).	Not mentioned.	Electrochemical sensing via SWV with application to real serum samples.	2020 [207]
Small cell lung cancer biomarkers					
ProGRP	Epitope- bulk polymerisation.	EAMA, DVB& EGDMA, ADVN, DMSO.	0.5 mg mL^{-1}	Developed MIPs were used as SPE for extraction and pre-concentration of ProGRP prior to LC-UV analysis without application to biological samples.	2014 [208]
ProGRP	Epitope-core shell surface imprinting on silica beads (RAFT).	EAMA, DVB& EGDMA, ADVN, ACN: DMSO (80:20 v/v).	625 pM	Developed MIPs were used as SPE for extraction and pre-concentration of ProGRP prior to LC-MS/MS analysis with application to spiked serum samples.	2014 [209]
ProGRP	Epitope-precipitation polymerisation (RAFT).	EAMA, DVB, AIBN, ACN.	17.2 pM	Developed MIPs were used as SPE for online extraction and pre-	2017 [210]

				concentration of ProGRP prior to LC-MS/MS analysis with application to real serum samples.	
ProGRP	Bulk polymerisation.	MIP1: MAA, EGDMA MIP2: EAMA, DVB (better performance).	Not mentioned	Developed MIPs were used as SPE for extraction and pre-concentration of ProGRP prior to LC-MS/MS analysis without application to biological samples.	2017 [211]
ProGRP	Epitope-precipitation and core shell surface imprinting (magnetic).	Different types of magnetic core−shell polymer microspheres and magnetized polymer microspheres were synthesized with different set of reagents.	39 pM	Developed MIPs were used for extraction and magnetic separation of ProGRP prior to LC-MS/MS analysis with application to spiked serum samples.	2020 [212]
NSE	Epitope-electropolymerisation on gold wire and QCM chip.	Scopoletin electropolymerised using two different epitopes and initiation was done via multistep amperometry.	1 ng mL^{-1} 15.6 nM for the two epitope imprints.	Electrochemical determination of NSE without application to biological samples.	2018 [213]
NSE	Precipitation polymerisation.	1,2,2,6,6-pentamethylpiperidine & N-3,5-bis(Trifluoromethyl)-phenyl-N'-4-vinylphenylurea, DBV, AIBN, ACN:THF (8:1 v/v).	1.8 ng mL^{-1}	Developed MIPs were used as SPE for extraction and pre-concentration of NSE prior to LC-MS/MS analysis with application to spiked serum samples.	2020 [214]

NSE	Epitope-electropolymerisation on gold wire.	Scopoletin in EtOH: 0.1M sodium chloride (1:19 v/v).	0.25 µM	Electrochemical sensor for determination of NSE. with application to spiked serum samples.	2020 [215]
NSE	Epitope-electropolymerisation with gold NPs on gold electrode.	Scopoletin electropolymerised with hybrid of two epitopes.	25 pg mL^{-1}	Electrochemical sensor applied to spiked serum samples.	2020 [216]
Breast cancer biomarkers					
CA 15-3	Electropolymerisation on screen printed gold electrode.	2-aminophenol and initiation was done via CV in phosphate buffer (pH 7.4).	1.5 U mL^{-1}	Electrochemical sensor applied to spiked serum samples.	2017 [217]
CA 15-3	Electropolymerisation on screen printed gold electrode.	Toluidine blue and initiation was done via CV.	0.1 U mL^{-1}	Electrochemical sensor applied to spiked serum samples.	2018 [218]
CA 15-3	Electropolymerisation on fluorine doped tin oxide conductive glass support.	Pyrrole and initiation was done via CV in Hepes buffer (pH 6.5).	1.07 U mL^{-1}	Potentiometric electrochemical sensor applied to synthetic serum samples.	2018 [219]
HER2-ECD	Electropolymerisation on screen printed gold electrode.	Phenol and initiation was done via CV in phosphate buffer (pH 7.4).	1.6 ng L^{-1}	Electrochemical sensor applied to spiked serum samples.	2018 [220]
Colorectal cancer biomarkers					
CEA	Electropolymerisation on silver screen printed electrode.	Pyrrole and initiation was done via CV.	Not mentioned.	Electrochemical detection and quantitation of CEA	2016 [221]

				with application to spiked urine samples.	
CEA	Electropolymerisation on fluoride-doped tin oxide glass.	Pyrrole and initiation was done via CV in PBS buffer (pH 7.4).	0.13 pg mL^{-1}	Electrochemical detection and quantitation of CEA with application to spiked urine samples.	2018 [222]
CEA	Electropolymerisation on fluoride-doped tin oxide glass.	Aniline and initiation was done via CV in acetate buffer (pH 6.0).	0.1 pg mL^{-1}	EIS and CV electrochemical sensors applied to spiked urine samples.	2019 [223]
CEA	Electropolymerisation on poly(3,4-ethyle nedioxythiophene)/ fluoride-doped tin oxide glass.	Pyrrole and initiation was done via CV in PBS buffer (pH 7.4).	0.14 ng mL^{-1}	Electrochemical sensor applied to spiked urine samples.	2019 [224]
CEA	Electropolymerisation on microfluidic paper device.	Dopamine and initiation was done via CV.	0.32 ng mL^{-1}	Electrochemical sensor applied to real serum samples.	2019 [225]
CEA	Electropolymerisation on a hollow gold nanoballs/$MoSe_2$ nanosheets modified glassy carbon electrode.	3-{[{4-N,N-Bis[(carbamoyl)-ethylmethacrylate] butyl}((carbamoyl) amino)ethyl methacrylate] -propyl}- 1-ethenyl-1H-imidazol-3-ium bromide ionic liquid, MBA, TEMED & APS, phosphate buffer (pH 7.4).	11.2 pg mL^{-1}	Electrochemical sensor applied to real serum samples.	2019 [226]
CEA	Electropolymerisation on fluorine doped tin oxide glass modified with a homemade carbon ink.	Aminophenol & aminophenylboronic acid and initiation was done via CV in acetate buffer (pH 5.0).	2 ng mL^{-1}	Electrochemical detection and quantitation of CEA with application to spiked fetal bovine serum.	2019 [227]

CEA	Surface imprinting on surface Raman spectroscopy substrate.	Dopamine in Tris buffer (pH 8.5).	0.06 pg mL^{-1}	Raman spectroscopic sensor applied to real serum samples.	2019 [228]
CEA	Electropolymerisation on fluorine doped tin oxide glass.	EDOT and pyrrole .	0.08 ng mL^{-1}	Electrochemical sensor applied to spiked serum samples.	2021 [229]
Hepatic cancer biomarkers					
AFP	Core shell surface imprinting on QDs (fluorescent).	Vinylphenylboronic acid & γ-methacryloxypropylmethacrylate, APS, carbonate buffer (pH 9.0).	48 ng L^{-1}	Fluorescent detection and quantitation of AFP with application to real serum samples.	2015 [230]
AFP	Core shell surface imprinting on CDs (fluorescent).	2-acryloylamino-pentanedioic acid & NIPAM, MBA, APS, DMSO.	0.42 ng mL^{-1}	Fluorescent detection and quantitation of AFP with application to serum, plasma and urine samples.	2016 [231]
AFP	Core shell surface imprinting on CDs (fluorescent).	NIPAM & 4-vinylphenylbronic acid, MBA, ammonium peroxodisulphate.	0.474 ng mL^{-1}	Fluorescent nanosensor applied to spiked serum samples.	2019 [232]
AFP	Surface imprinting on a microscopic glass slide.	3-(2-azido-acetylamino)phenylboronic acid & propargyl acrylate ,TEOS, AIBN, phosphate buffer (pH 7.4).	9.65 ng mL^{-1}	Fluorescent detection and quantitation of AFP with application to spiked serum samples.	2019 [233]
AFP	Surface imprinting on gold nanoparticle modified glassy carbon electrode.	1-[3-(N-cystamine)propyl]-3-vinylimidazolium tetrafluoroborate ionic liquid, EGDMA, APS & TEMED, Tris buffer (pH 7.4).	2 pg mL^{-1}	Electrochemical detection and quantitation of AFP without application to biological samples.	2019 [234]

AFP & insulin	Core shell surface imprinting on aptamer DNA functionalised magnetite (magnetic).	TEOS, PBS buffer.	0.5 ng mL^{-1} for insulin Not mentioned for AFP	Nanorpobe for extraction of targets prior to MS analysis with application to real serum and saliva samples.	2020 [235]
Ovarian cancer biomarker					
CA-125	Electropolymerisation on gold nanoelectrode.	Phenol and initiation was done via CV in PBS (pH 7.4).	0.5 U mL^{-1}	Electrochemical detection and quantitation of CA-125 with application to spiked serum samples	2012 [236]
CA-125	Dummy-surface imprinting on CNTs.	Fluorescent metal chelating monomer: methacryloyl antipyrine terbium (III), EGDMA, 2,2-dimethoxy-2-acethophenone, ACN: water (1:1 v/v).	0.49 U mL^{-1}	Fluorescent nanosensor applied to spiked serum samples.	2017 [237]
CA-125	Electropolymerisation on gold electrode.	Pyrrole and initiation was done via CV in phosphate buffer (pH 7.2).	0.01 U mL^{-1}	Electrochemical sensor applied to spiked artificial serum samples.	2019 [238]
Miscellaneous cancer biomarkers					
Galectin-3	Electropolymerisation on carbon screen printed electrode.	3-Aminophenol in PBS buffer	Not mentioned	EIS sensor applied to spiked serum samples.	2021 [239]
Hyaluronan-linked protein 1	Surface imprinting on gold silicone chip.	Self-assembly of 11-mercapto-1-undecanol in deionized water: EtOH (19:1 v/v).	Not mentioned.	Electrochemical detection and quantitation of target with application to spiked serum samples.	2013 [240]
Kininogen fragments (K-2209 & K-1944)	Epitope-solid phase synthesis on porous silica beads.	4-VP, EGDMA, AIBN, ACN and copper acetate.	Not mentioned.	Developed MIPs were used for targets extraction and	2016 [241]

				enrichment prior to HPLC analysis with application to spiked serum samples.	
Regenerating Protein 1B	Epitope-surface imprinting via solvent evaporation on screen printed gold substrate.	Ethylene-co-vinyl alcohol, DMSO.	0.1 pg mL^{-1}	Electrochemical detection and quantitation of target with application to real urine samples. In latter work, the authors used magnetic MIPs to extract the target from cell cultures before electrochemical sensing.	2017, 2018 [242,243]
VEGF	Epitope-solid phase synthesis on glass beads with associated QDs.	NIPAm, AA, N-tert-butylacrylamide& N-(3-aminopropyl) methacrylamide, MBA, TEMED & APS, PBS (pH 7.4).	Not mentioned	Fluorescent sensor for detection and quantitation of VEGF, applied to malignant melanoma cells in zebrafish embryos.	2017 [244]
EGFR and VEGF	Surface imprinting on gold screen printed electrode.	Am, MBA, APS, PBS (pH 7.4).	0.01 & 0.005 pg mL^{-1} for EGFR and VEGF.	Potentiometric striping analysis combined with liposomal amplification strategy for determination of targets with application to real serum samples.	2018 [245]
VEGF	Electropolymerisation on graphite screen printed electrode.	o-phenylenediamine and initiation was done via CV	0.08 pg ml^{-1}	EIS electrochemical sensor applied to spiked serum samples.	2019 [246]

Mettalothionein	Bulk polymerisation (self-assembly).	Dopamine in Tris buffer (pH 8.5).	Not mentioned.	Developed MIPs were used as SPE for extraction and pre-concentration of target prior to MS analysis with application to spiked serum samples.	2018 [247]
Mettalothionein	Core shell surface imprinting (magnetic)	(8.5 pH) buffer Tris in Dopamine	Not mentioned	Developed MIPs were used for target extraction and enrichment prior to analysis via laser ablation inductively coupled plasma coupled to mass spectrometry with application to pig skin samples.	2020 [248]
Cardiac diseases biomarkers					
Troponin T	Electropolymerisation on gold electrode.	o-phenylenediamine and initiation was done via CV in acetate buffer (pH 5.2).	9 pg ml^{-1}	Electrochemical detection and quantitation of target with application to spiked serum samples.	2013 [249]
Troponin T	Electropolymerisation on graphene oxide screen printed electrode.	Pyrrole-3-carboxylic acid & pyrrole in $LiClO_4$ solution in phosphate buffer (pH 5.8).	0.006ng mL^{-1}	Electrochemical detection and quantitation of target via DPV & CV with application to real serum samples.	2016 [250]

Troponin T	Epitope-surface imprinting on SPR gold chip.	Dopamine in Tris buffer (pH 8.5).	15.4 nM	SPR detection and quantitation of target with application to spiked serum samples.	2018 [251]
Troponin T	Electropolymerisation on gold electrode.	o-phenylenediamine in acetate buffer (pH 5.2).	0.017 ng mL^{1-}	Linear sweep voltammetric detection and quantitation of target with application to clinical serum samples.	2020 [252]
Troponin T	Electropolymerisation on screen printed electrodes modified with MWCNTs.	Aniline in phosphate buffer (pH 5.8).	0.040 pg mL^{-1}	Electrochemical detection and quantitation of target via DPV with application to real serum samples.	2020 [253]
Troponin I	Epitope-bulk polymerisation.	Itaconic acid, trimethylolpropane-trimethacrylate, 2,2-dimethoxy-2-phenylacetophenone, water or DMSO.	300 femtomoles	MIPs were used for extraction and enrichment of target prior to MALDI-TOF-MS analysis with application to serum mimicking samples.	2015 [254]
Troponin I	Electropolymerisation on glassy carbon electrode.	o-aminophenol and initiation was done via CV in $NaClO_4$ (pH 7.0).	0.027 nM	Electrochemical detection and quantitation of target via EIS with application to spiked serum samples.	2016 [255]
Troponin I	Epitope-surface imprinting on SPR gold chip.	Norepinephrine in Tris buffer (pH 8.5).	7.1 ± 0.6 nM	SPR detection and quantitation of target with application to	2020 [256]

				spiked serum samples.	
Myo	Electropolymerisation on gold screen printed electrode.	o- aminophenol and initiation was done via CV in acetate buffer (pH 5.0).	1.5 & 0.8 µg mL^{-1}	Electrochemical detection and quantitation of Myo via SWV and EIS with application to spiked serum samples.	2014 [257]
Myo	Surface imprinting on graphite screen printed electrode.	4-tyrenesulfonic acid & 2-aminoethyl methacrylate hydrochloride, EGDMA, BPO, o-nitrophenyloctyl Ether.	0.79 µg mL^{-1}	Electrochemical detection and quantitation of Myo via SWV with application to spiked urine samples.	2014 [258]
Myo	Electropolymerisation on CNTs modified screen printed electrode.	o-phenylenediamine in acetate buffer (pH 5.2).	Not mentioned	Electrochemical detection and quantitation via CV & SWV with application to serum samples.	2016 [259]
Myo	Electropolymerisation on gold screen printed electrode.	Phenol and initiation was done via CV in PBS (pH 7.0).	2.1 pg mL^{-1}	Electrochemical detection and quantitation via SWV with application to spiked serum samples.	2017 [260]
Myo	Suspension polymerisation.	N-methacryloylamino folic acid-Nd^{3+} in DMSO, EGDMA in toluene, AIBN, aqueous dispersion phase of poly(vinyl alcohol).	Not mentioned	Circular dichroism spectroscopic determination of Myo with application to real serum samples.	2018 [261]
Myo	Surface imprinting on QDs (fluorescent) and bulk polymerisation.	2-aminoethyl methacrylate hydrochloride & Am, MBA, TEMED & APS.	0.045 pg ml^{-1}	Fluorometric detection and quantitation of Myo with application to	2018 [262]

				spiked serum samples.	
Myo	Bulk polymerisation (hydrogels).	Different Am monomers, MBA, TEMED & APS, water.	N/A	Detection of Myo with no analytical application.	2019 [263]
Myo	Bulk polymerisation (fluorescent hydrogels).	Am & fluorescein o-acrylate, MBA, TEMED & APS, water.	60 pg m^{-1}	Fluorometric detection and quantitation of Myo with application to spiked fetal calf serum.	2021 [264]
Myo	Core shell surface imprinting on quantum dots (fluorescent).	Am, MBA, APS & TEMED, PBS buffer (pH 6.4).	3.08 pg mL^{-1}	Fluorescent sensor for detection and quantitation of Myo with application to real serum samples.	2021 [265]
Heart-fatty acid binding protein	Electropolymerisation on gold nano/micro-islands/ chemically reduced graphene oxide.	o-phenylenediamine in acetate buffer (pH 5.2).	2.29 fg mL^{-1}	Electrochemical sensor applied to spiked serum samples.	2021 [266]
Angiotensin(II)	Cryopolymerisation at -16 °C.	1-Vinylimidazole & HEMA, MBA, APS & TEMED, water.	Not mentioned	The developed spongy MIPs were used for extraction of angiotensin (II) directly from crude human serum.	2020 [267]
Neurological diseases biomarkers					
Dopamine neurotrophic factor protein	Electropolymerisation on sensor chip surface.	m-phenylenediamine in PBS buffer.	4.2 ng ml^{-1}	Surface acoustic wave sensor applied to biological samples.	2020 [268]
β-Amyloid-42	Electropolymerisation on polyaniline coated	α-CDs in PBS buffer.	0.20 ng mL^{-1}	Electrochemical sensor via EIS and SWV applied to fetal	2017 [269]

	gold screen printed electrode.			bovine serum samples.	
β-Amyloid-42	Electropolymerisation on carbon electrode modified with CNTs doped with copper NPs.	Aniline in PBS buffer (pH 7.2).	0.40 pg/mL	Electrochemical sensor via SWV applied to artificial serum samples.	2018 [270]
β-Amyloid-42	Electropolymerisation on carbon-ink electrodes prepared on a paper support.	o-phenylenediamine and initiation was done via CV in PBS (pH 7.2).	0.067 ng mL^{-1}	Electrochemical detection and quantitation of target with application to fetal bovine serum samples.	2020 [271]
β-Amyloid-42	Electropolymerisation on delaminated titanium carbide and MWCNTs modified glassy carbon electrode.	Pyrrole and initiation was done via CV in PBS (pH 7.5).	0.3 fg mL^{-1}	Electrochemical detection and quantitation of target with application to spiked plasma samples.	2020 [272]
Plasma Iron levels biomarker					
TrF	Solid phase synthesis on porous silica particles.	MAA & methacrylamide, 1,4-bis(acryloyl)piperazine & ammonium sulphate, PBS (pH 7.4): ACN (4:1 v/v).	Not mentioned	Prepared MIPs were used for recognition and extraction of TrF without application to biological samples.	2013 [273]
TrF	Surface imprinting on gold nanorods.	Dopamine in Tris buffer (pH 7.5).	10^{-8} M	Plasmonic nanosensor coupled to SERS for detection and quantitation of TrF with application to spiked serum samples.	2016 [274]
TrF	Core shell surface imprinting (magnetic).	DES (choline chloride + AA), MBA, APS & TEMED, phosphate buffer (pH 5.0).	Not mentioned	Prepared MIPs were used for extraction and	2018 [49]

				pre-concentration of TrF prior to UV analysis with application to spiked serum samples.	
TrF	Core shell surface imprinting (magnetic) with post imprinting. fluorescent modification.	TEOS, ammonia & EtOH.	0.0075 mg mL^{-1}	Fluorometric detection and quantitation of TrF with application to real serum samples.	2018 [275]
TrF	Electropolymerisation on gold wires or gold SPR chip.	Scopoletin in sodium chloride solution.	Not mentioned	Electrochemical and SPR detection and quantitation of TrF without application to biological samples.	2018 [276]
TrF	Electrodeposition on gold coated QCM.	Starch NPs-reduced graphene oxide nanocomposites, glutaraldehyde, water and initiation was done via CV.	20 ppb	Electrochemical detection and quantitation of TrF with application to real plasma samples.	2019 [277]
Metabolic disorders biomarkers					
Cytochrome c & lysozyme	Bulk polymerisation (hydrogel).	2-Acrylamido-2-methyl-propanesulfonic acid & sodium allylsulfonate & sodium p-styrenesulfonate, polyethylene glycol dimethacrylate, 2,2′-azobis[2-(2-imidazolin-2-yl)propane], Tris buffer (pH 7.0).	N/A	Developed MIPs were used for selective adsorption of targets with no analytical application.	2015 [278]
Cytochrome c	Epitope-core shell surface imprinting (metal coordination) on silicon NPs (fluorescent).	Zinc(II) acrylate, EGDMA, AIBN, dimethylformamide.	0.32 µM	Fluorescent sensor for detection and quantitation of target, applied to spiked human serum and fetal bovine plasma.	2018 [279]

Cytochrome c	Cryopolymerisation at -20 °C.	N-methacryloylamido antipyrine-Ce(III) & HEMA, MBA, APS & TEMED, water.	Not mentioned	Developed MIPs were used for selective adsorption of cytochrome c and applied to spiked serum samples.	2021 [280]
Miscellaneous biomarkers					
β2-micro-globulin (many diseases)	Epitope-core shell surface imprinting on silica beads.	MAA, N,N-ethylenebis(acrylamide), APS, PBS buffer: ACN (1.5:0.2 v/v).	0.058 mg mL^{-1}	Developed MIPs were used as SPE for online extraction and pre-concentration of target prior to HPLC analysis with application to spiked serum samples.	2017 [281]
Serum C-terminal telopeptide of type I collagen (Bone loss)	Precipitation polymerisation.	MAA, EDGMA, AIBN, ACN.	0.09 ng mL^{-1}	Interdigital capacitive sensor combined with EIS for determination of target with application to sheep serum samples.	2018 [282]
Oxytocin (autism)	Electropolymerisation on gold film electrode.	4-bis(2,2'-bithien-5-yl)methylbenzoic acid glycol ester, 2,4,5,2',4',5'hexa(thiophen-2-yl)-3,3'-bithiophene, tetrabutylphosphonium tetrafluoroborate.	60 µM	Microfluidic EIS sensor applied to synthetic serum samples.	2018 [283]
Pepsin (gastric reflux)	Mini-emulsion polymerisation.	MAA/HEMA, EGDMA, 2,2dimethoxy-2-phenyl-acetophenone, n-hexadecane Lutensol and pepsin in water phase	N/A	Prepared MIPs were used for recognition and extraction of pepsin without analytical application.	2013 [284]
Pepsin (gastric reflux)	Solid phase synthesis on glass beads with magnetic element.	N-tert-butylacrylamide & NIPAm & N-(3-aminopropyl) methacrylamide	Not mentioned.	Microplate-based assay equipped with magnetic inserts and	2018 [2]

		hydrochloride & AA, MBA, TEMED & PPS, water.		polystyrene beads used as fluorescent coloured label for detection of pepsin with application to synthetic gastric juice samples.	
Non-structural protein 1 (Dengue fever viral infection)	Surface imprinting on screen printed carbon electrode modified with electrospun nanofibers of polysulfone.	Dopamine in Tris buffer (pH 8.5).	0.3 ng mL^{-1}	Impedimetric sensor applied to spiked serum samples.	2020 [285]
Heat-denatured non-structural protein 1 (Dengue fever viral infection)	Hidden epitope-electropolymerisation on carbon screen-printed electrode.	and (7.4 pH) buffer PBS in Aminophenol CV via done was initation	29.3 µg L^{-1}	EIS sensor applied to spiked serum samples.	2021 [286]
Interleukin-2 (inflammations and cancers)	Core shell surface imprinting on QDs (fluorescent).	MAA, MBA, TEMED & APS, PBS (pH 6.5).	5.91 fg ml^{-1}	Fluorometric detection and quantitation of target with application to spiked synthetic serum samples.	2020 [287]
Leukotriene-4 & insulin	Solid phase synthesis on glass beads.	Itaconic acid, allylamine & fluorescein o-acrylate, trimethylolpropane trimethacrylate & EGDMA, diethyldithiocarbamic acid benzyl ester. Itaconic acid, allylamine, fluorescein o-acrylate & NIPAm, MBA, APS & TEMED.	0.73 pM & 27 pM	Microplate-based assay for detection and quantitation of targets with application to spiked artificial urine and blood plasma.	2020 [288]
Fibrinopeptide B (venous thromboembolism)	Surface imprinting on opal photonic structure made of	MAA, MBA, TEMED & APS, 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.0).	0.13 ng mL^{-1}	Optical reflectance sensor applied to spiked urine samples.	2020 [289]

	self-arranged silica Nps.				
Ovalbumin (allergy)	Suspension polymerisation.	Amphiphilic fluorescent monomer made of: vinylphenylboronic acid, AA and N-vinyl carbazole, and modified with glycidyl methacrylate and initiation was done via UV light.	0.033 pg mL^{-1}	Fluorescent sensor for detection and quantitation of target, applied to hen eggs and commercial products.	2020 [290]
Cystatin C (chronic renal diseases)	Electropolymerisation on carbon screen-printed electrode.	Pyrrole-3-carboxylic acid & pyrrole in acetate buffer (pH 6.0).	0.5 ng mL^{-1}	Electrochemical sensor via CV and DPV applied to spiked serum samples.	2021 [291]
Chymotrypsinogen (acute kidney failure)	Surface imprinting on a microfluidic paper.	(8.5 pH) buffer Tris in Dopamine	3.5 μM	Microfluidic paper device for visual detection and quantitation of chymotrypsinogen via fluorescent derivatizing agent and applied to spiked urine samples.	2021 [292]

As mentioned earlier, we classified the reviewed methods based on the disease. For a specific disease, we start by outlining a scientific critique of the methods applied for this particular disease's biomarkers.

4.1. Cancer biomarkers

4.1.1. Prostate cancer biomarkers

Prostate specific antigen (PSA) is considered the primary biomarker used up till now for diagnosis and follow up of prostate cancer patients. PSA is a bulky glycoprotein of molecular weight ~ 30 kDa, which is found in serum and plasma samples [293]. We found the first paper applying MIPs combined with a sensing technique for analysis of PSA published in 2014. The method relied on the use of MIPs as plastic antibodies for detection and quantification of PSA via a potentiometric sensor. Synthesis of MIPs depended on template immobilisation to graphene sheets via covalent bonds. The covalent immobilisation enabled easy template removal and better orientation of binding sites. Moreover, researchers used charged monomers: vinyl benzoate (positive quaternary ammonium salt) and (vinylbenzyl)trimethylammonium chloride (negative polarity) to increase the interaction with PSA. Even though the use of charged monomers can cause an increase in non-specific binding, the authors optimised and controlled the exact amount of charged monomers to prevent undesirable binding. The notable feature in this paper, is the use of trypsin enzyme to remove the template after polymerisation. Use of trypsin may cause partial blocking of binding sites by residual protein fragments and requires excessive washing cycles to remove residual fragments and the enzyme. Later in 2016, the authors of this paper introduced an application of their method for analysis of PSA in different cell culture lines, which contained different concentrations of PSA and other metabolites. The method showed an outstanding performance in the analysis of PSA in different samples with minimal interference from the coexisting metabolites [201].

A unique combination of MIPs and biological recognition elements has been presented in an electrochemical EIS sensor to determine PSA [202]. The method used a DNA aptamer with high affinity for PSA combined with MIPs layer of poly(dopamine) on the surface of gold electrode. Both MIPs and aptamer acted synergistically to enhance sensitivity of the method as LOD reached 1 pg mL^{-1}. Moreover, dopamine was chosen for electropolymerisation, since it polymerises at low potential, which eliminates the risk of oxidation of the DNA thiol link employed in immobilisation to the surface of the electrode. However, the authors mentioned reduced selectivity of the sensor compared to an aptamer only sensor. They attributed the decreased selectivity to the embedment of part of the aptamer into the poly(dopamine) layer, which covered some of its binding sites and increased nonspecific, non-covalent binding with the poly(dopamine) MIPs layer. We also like to point out a concern of stability of the DNA aptamer on the long-term use. Although some researchers debate that stability of the aptamer increases after inclusion in the polymer [294], yet it is still susceptible to enzymatic degradation by nucleases [295].

CRP techniques have been applied for synthesis of MIPs specific for PSA. A RAFT technique was applied combined with the use of hydrophilic monomer for the synthesis of MIPs coupled to LOVE wave acoustic sensor. The use of hydrophilic monomer increased selectivity by minimizing nonspecific interactions and increasing binding affinity in addition to increasing applicability to aqueous media samples [203]. On the other hand, an ATRP technique was used in another paper to synthesis MIPs for PSA coupled to a SPR sensor. The authors of this paper added a post imprinting modification to the resulting MIPs using polyethylene glycol

based capping agents to cover the low affinity binding sites [204]. In both papers, sensitivity and selectivity were significantly enhanced in addition to the previously mentioned advantages of CRP.

There is some disagreement regarding the washing solutions and techniques for complete removal of template from MIPs after polymerisation. There is no single reagent that can be effective for removal of all protein templates, therefore optimisation of the washing step is necessary to ensure complete extraction of analytes from their binding sites. Moreover, care should be taken that the washing solution does not adversely affect the stability or the performance of the analytical method [296]. Z. Yazdania et al. prepared an electrochemical sensor for PSA via electropolymerisation of pyrrole on screen-printed gold electrode [297]. Various solutions were tested in this paper to choose the optimum washing solution to remove PSA template based on recommendations from precedent papers and, oxalic acid solution was shown to be the most effective. This illustrates the need for a study of the washing step, which we do not normally observe in many of the published papers.

A very clever combination of MIPs recognition abilities and antibodies recognition have been demonstrated in a dual template electrochemical sensor for determination of PSA and myoglobin. The method adopted the formation of MIPs with dual template identification properties through the self-assembly of 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide ester)) on a screen printed electrode in the presence of the two templates (ratio 1:1). EIS was used to determine the electrochemical signal produced from both templates upon binding. Afterwards, magnetic nanocomposites composed of magnetite, MWCNTs, graphite and PSA antibodies were added to the sample bound to MIPs layer. The PSA antibodies present in the nanocomposites specifically bound to PSA resulting in another EIS signal and therefore, the Myo signal was calculated by difference. This technique is very innovative in its ability to utilize two different recognition techniques to simultaneously detect two biomarkers with no interference [298].

As mentioned before, a computational approach has been very useful to depict template-monomer interactions and allow for optimisation of monomer's type and concentration to reduce the overall number of optimisation trials. Visual Molecular Dynamics software was used to study molecular dynamics between pyrrole and PSA. The molecular dynamics calculations in this study showed very strong binding between PSA and the polypyrrole network in whatever starting orientation of molecules. Moreover, the most probable orientation of PSA to pyrrole molecules showed the least adsorption energy (Gibbs energy) and highest contact area, which was indicative of high stability and binding [299]. This emphasize that computational techniques can save much time and effort in addition to providing numerical proof of binding and stability.

4.1.2. Small cell lung cancer (SCLC) biomarkers

Progastrin releasing peptide (ProGRP) is a well-established biomarker that is used to diagnose and monitor patients of SCLC [300]. ProGRP signature peptide; a peptide sequence of 9 amino acids (epitope) was used as a template for MIPs synthesis intended for SPE separation prior to analysis [208]. As a method of optimisation, researchers carried out a combinatorial library of all possible MIPs compositions with different functional monomers and cross linkers. A total of 96 MIPs were prepared by an automated auto-sampler and were investigated for their affinity and selectivity. The best polymer composition involved the use of DVB as a cross linker. However, the use of DVB led to a hydrophobic nature of the resulting polymer and therefore, reduced its applications in aqueous media. In addition, the

hydrophobic nature increased the nonspecific binding with other amino acids sequences in the protein sample digest and required the use of organic modifiers. Moreover, bulk imprinting technique employed in this paper is contraindicated in SPE applications. Later in the same year, another group of researchers adopted the same published composition of MIPs, however, they used the RAFT polymerisation technique [209]. RAFT polymerisation significantly enhanced the properties of MIPs and reduced their size distribution. Furthermore, the authors understood the problem associated with the hydrophobic nature of MIPs and they provided an extensive study on the washing step to minimize nonspecific binding. The authors scrutinized different ratios of aqueous solution of ACN as washing solution to reach the optimum composition. We would also like to point out the enhanced sensitivity of this method (LOD = 625 pM) compared to the former method, which can be attributed to the use of LC-MS that offered better sensitivity compared to HPLC-UV. Later in 2017, a further improvement was made to almost the same composition of MIPs introduced in the two previous papers [210]. A precipitation polymerisation technique combined with RAFT was successfully utilized to produce more uniform, spherical and small sized MIPs microspheres. In addition, the authors upgraded the SPE system to an online SPE coupled to LC-MS for a faster and more efficient loading and minimum loss of sample. This upgrade significantly enhanced the sensitivity to a level comparable to the expected serum levels of ProGRP. Further addition to this method was performed in which of SPE conditions were carefully investigated using an automated 384-well filter plate coupled to matrix-assisted laser desorption/ionisation (MALDI) ion source to determine the best composition [211].

Another equally important protein biomarker for SCLC is neuron specific enolase (NSE), a 45 kDa protein that is highly specific for neurons and neuroendocrine cells and is found in different body fluids of SCLC patients [301]. Three reported electrochemical methods in our literature survey applied the epitope approach for the analysis of NSE. The three methods are quite similar in both the utilized electrochemical technique and in the use of the same functional monomer (scopoletin) for synthesis of MIPs films on the working electrode. However, each method used a unique epitope as target. In the method reported in 2018 [213], the authors utilized two different epitopes: cysteine and histidine modified epitopes to prepare two different sensors and compare their performance. Results of this paper demonstrated that the sensor made using a histidine modified epitope was superior in affinity, sensitivity and performance to the sensor made using the cysteine modified epitope, as indicated by the higher imprinting factor (11) and better LOD (15.6 nM). Later in 2020, the second electrochemical method utilized a double cysteine modified epitope for molecular imprinting [215]. The double cysteine modified epitope resulted in two assembly points of the epitope with the electrode surface. This form of assembly resulted in template bridges, which were reflected as horizontal cavities with more binding sites on the surface of MIPs compared with the vertically aligned single modified epitope template. This modification was supposed to increase sensitivity, however comparing the LOD values, the previous method has a superior sensitivity compared to the newer one (0.25 µM). The third published electrochemical method also in 2020 utilized two approaches to improve the performance and sensitivity to a very high level [302]. The authors of this paper used two different cysteine modified epitopes and gold NPs in their MIPs structure. The use of two different epitopes allowed for more and versatile identification points in MIPs binding sites. The use of two epitopes can be very challenging, since it is difficult to prevent inter-epitope cross linking, yet researchers carefully optimised the voltage required for the oxidation of cysteine thiol bond. This was necessary, since other voltages could favour epitopes cross linking. In addition, gold NPs acted as signal amplifying agents to boost the binding signal, hence sensitivity was improved 20-fold compared with

sensors without the gold NPs. This electrochemical method was the most successful in terms of sensitivity reaching to comparable biological levels of NSE where the linear range was (200 to 4000 pg mL^{-1}) and LOD was (25 pg mL^{-1}).

4.1.3. Colorectal cancer biomarkers

Carcinoembryonic antigen (CEA) is considered the main biomarker used currently for the diagnosis of colorectal cancer. CEA normal level in adults is usually below 3 ng mL^{-1}, however higher levels are usually a biomarker for colorectal cancer [303]. The very low concentration of this biomarker requires the use of sensitive and highly efficient techniques for its quantitation. This explains why most of the methods in our literature survey are electrochemical methods, since they usually offer a very high level of sensitivity. Dye sensitized solar cells (DSSC) are a type of thin film solar cells, which draw their power from solar (light) energy. This type of cells offers many advantages the most important of which are environmental friendliness, low toxicity, and simple setup [304]. DSSC technology was first combined with molecular imprinting to produce an electrochemical platform for the analysis of CEA. The DSSC setup is composed of a working electrode, which contains the dye adsorbed onto TiO_2 NPs and spread on a glass substrate, an electrolyte solution and a counter electrode. A layer of polypyrrole was electropolymerised onto the counter electrode as a biorecognition element for selective binding of the target protein (CEA). The developed method was highly sensitive where LOD reached 0.13 pg mL^{-1} and there was no need for an external electrical source or electrical reading box or cables, therefore it was suitable as a portable device and as a point of care analytical method [222]. One year after this publication, two of the authors repeated the same method for the same target protein, however using a different monomer for MIPs deposited on the counter electrode. The authors claimed that the first method had little power, which in turn reduced efficiency, sensitivity and linear range of the method. Consequently, the authors used polyaniline MIPs as a more conductive material for molecular imprinting to coat the counter electrode instead of polypyrrole. Despite this alteration, there was no significant enhancement in sensitivity where LOD was 0.10 pg mL^{-1} and the linear range was not improved either [305]. Later in 2019, the same authors applied a modification to their own method for determination of CEA [224]. An electropolymerised layer of 3,4-ethylenedioxythiophene (EDOT) was introduced to the counter electrode between the glass substrate and the polypyrrole MIPs layer. The poly(EDOT) layer conferred an electrochromic nature to the DSSC setup. Upon binding of CEA, the electrochromic cell changed colour in a concentration dependent manner. This was a significant improvement for the method, since the response was visual and could be semi-quantitatively linked to the concentration of CEA in samples for easy readout. Moreover, despite the low sensitivity of the method, the LOD and linear range still lied within the CEA concentration range anticipated in body fluids. However, the authors used pyrrole again for the synthesis of MIPs layer despite their previous claim that aniline is a better alternative. Nonetheless, we like to point out that DSSC is still limited by some challenges that need improvement prior to their involvement in analytical methods. There are different concerns regarding the stability of DSSC that are discussed in detail in this cited paper [304]. These concerns need to be addressed in future research work using DSSC in analysis of such important biomarkers.

As another alternative to DSSC transducer in electrochemical methods, a passive direct methanol fuel cell was coupled to an electrochemical sensor setup for determination of CEA [229]. The new method showed a wider linear range and better sensitivity compared to the last published paper using DSSC. In addition, the passive direct methanol fuel cell has a simple design, high efficiency and low to zero emissions [306].

A novel method has been adopted recently for analysis of CEA, which employed the miniaturized method of paper-based microfluidic systems. Paper microfluidic chips are a type of microfluidic systems that uses hydrophilic paper materials (cellulose or nitrocellulose) to act as tubes to guide a fluid from an inlet portal to the desired outlet by a type of diffusion force called imbibition. Paper-based microfluidics provide many advantages such as low cost of synthesis, light weight, ease of disposability, green synthesis and portability [307]. Electropolymerisation of dopamine on the working electrode produced a MIPs layer selective for CEA. The smart design of the paper chip gathered all the necessary elements together, the synthesis and washing compartments along with the electrochemical detection elements were all on a 5 cm paper platform controlled by a movable valve and an origami method of folding. The microfluidic system only required very small volume of reagents, which enabled a continuous and easy delivery of fluid to carry out a multistep electropolymerisation over a long period of time. Despite the many merits associated with the involvement of microfluidics system, there is a limited sensitivity (LOD 0.32 ng mL^{-1}). This was attributed to the roughness of the surface of the working electrode due to the complex structure of paper fibres. However, the LOD is still significantly below the limit found in biological fluids, therefore this method has potential to be applied for point of care testing in real patients [225].

Away from the scope of electrochemical methods usually employed in the analysis of biomarkers, a new surface enhanced Raman spectroscopy (SERS) technique combined with surface molecularly imprinted polymers was recently published for the analysis of CEA [228]. Despite the high sensitivity of SERS reaching to a single molecule level, it suffers from some drawbacks, the most important of which are interference from endogenous substances producing signals in the fingerprint region (300-1800 cm^{-1}). Secondly, errors that occur due to variations in laser power and external environmental conditions resulting in fluctuations in SERS peaks. In an attempt to alleviate these limitations, the authors used a unique Raman reporter with silent Raman signals encapsulated in nanocomposites to remove the optical noise in the fingerprint region. Moreover, to mitigate the errors from the device, an internal standard was used, which enhanced the accuracy and the reproducibility of the method. Furthermore, the performance of the method was boosted by introduction of double layered core shell NPs in the SERS substrate, which acted as hot spots for enhancement of electromagnetic response for SERS measurements. In spite of the complexity and multistep synthesis procedure of this SERS probe, the sensitivity was greater than the other reported papers in our survey for CEA (LOD= 0.064 pg mL^{-1}).

4.1.4. Hepatic cancer biomarker

Diagnosis of liver cancer relies on measuring the level of alpha fetoprotein (AFP) in serum. AFP is a 70 kDa glycoprotein that is normally found in plasma at low levels (between 10- 20 ng mL^{-1}), however this level is significantly increased to about 400 ng mL^{-1} in liver tumor patients [308]. Fluorescence quantitative analysis methods combined with the use of MIPs were widely used for the detection of AFP. The first method reported in 2016 was based on core shell surface imprinting on Mn-doped ZnS QDs with the use of a boronic acid derivative as functional monomer (vinylphenylboronic acid) [230]. This method is based on fluorescence enhancement after binding with target rather than fluorescence quenching. The authors explained the enhancement of fluorescence on the chemical basis of the bonds formed between AFP and the boronic acid monomer. AFP is a glycoprotein that has special cis diol groups, and the boronic acid monomer forms reversible covalent boronic ester bonds with AFP diol groups. In the absence of a target, fluorescence quenching occurs due to the electron transfer between QDs and boron moieties and loss of the excitation state. However,

after binding with AFP, MIPs become negatively charged, which disrupt the electrostatic interaction between QDs and boronic moieties leading to fluorescence enhancement. In addition, the covalent reversible bond increased the selectivity of MIPs and reduced nonspecific binding. In the same year, another fluorescence method was developed for determination of AFP, however the authors used CDs as the fluorescent element [231]. In this study, a thermo-responsive element, through the use of NIPAm, and a pH sensitive element, through the use of 2-acryloylamino pentanedioic acid functional monomers, were added. The advantages of these elements are that they allow accurate control of the temperature and the pH required for the optimum and highest binding capacity and also the pH and temperature required for release of template. When the temperature was below the low critical solution temperature (LCST) and at acidic pH, the MIPs structure was swollen and relatively loose to allow for maximum access for the target. However, on increasing temperature above LCST and at alkaline pH MIPs shrank, hydrogen bonds were disrupted, and binding was reduced dramatically. The authors reported that binding caused enhancement of fluorescence, yet they did not introduce an explanation for increased fluorescence instead of quenching. Later in 2019, a very similar paper was published using CDs, thermos-responsive and a pH sensitive element as well [232]. The pH sensitive monomer was 4-vinylphenylboronic acid, which aided in the formation of reversible covalent bonds with the target and boosted selectivity. The developed MIPs showed a similar behavior of temperature and pH-controlled binding, however alkaline pH was favourable for binding because it favoured the formation of boronic ester bonds with the cis diol group. In contrast to the previously published comparable method, this newer method depended on fluorescence quenching upon binding of target. Despite the fact that the use of boronic acid monomer was expected to cause fluorescence enhancement, similar to the first discussed paper for AFP, we believe this did not happen due the different nature of CDs and QDs. We presume that manganese doping of QDs facilitates the loss of electron to the boron moieties and fluorescence quenching in the absence of target. On the other hand, CDs do not contain transition metals to aid in electron loss. Therefore, binding to the target in this case decreases native fluorescence of CDs.

A different fluorescence method was introduced for determination of AFP in biological fluid samples that did not include a fluorescent core [233]. This method relied on fluorescence increase upon binding of AFP to a MIPs film formed on the surface of a microscopic glass slide. Herein, the method adopted the use of boronic acid monomers again for binding of AFP. The used monomer (3-(2-azido-acetylamino) phenylboronic acid) was polymerizable and fluorescent by itself. The authors attributed the fluorescence increase upon binding of target to the stabilization of the B-N bond between the boronic acid moieties and nitrogen atoms of the monomer. However, despite this, the authors criticized the use of core shell imprinted nanoMIPs for detection of trace amounts of biomarkers, claimed that large surface area was not needed, and their utility was limited. Their proposed method showed a very poor sensitivity (LOD = 9.65 ng mL^{-1}) compared to the previous two published methods using CDs.

4.1.5. Ovarian cancer biomarker

Cancer antigen-125 (CA 125) is a famous biomarker used for diagnosis and follow up on the prognosis of ovarian cancer patients [309]. Two very similar electrochemical methods have been published for determination of CA 125. Both methods relied on the electropolymerisation of pyrrole. However, in the first method published in 2012, MIPs layer was formed on the surface of 3D gold NPs ensembles [236], while the second method published in 2019, imprinting was performed on gold screen printed electrodes [238]. There was a dramatic

difference in sensitivity, the second method being far more superior (LOD 0.01 U mL^{-1}) compared to the first method (LOD 0.5 U mL^{-1}). We can only attribute the enhancement of sensitivity to the use of a screen printed electrode as working electrode, which usually shows very high conductivity and enhanced electrochemical performance.

Lanthanide elements usually have weak fluorescence characteristics that can be boosted when incorporated in metal chelated complexes [310]. It was this principle that researchers used to develop a new fluorescence sensor for determination of CA 125 [237]. The authors of this paper synthesized two metal chelating monomers: methacryloyl antipyrine terbium (III) and methacryloyl antipyrine europium (III) to be used in surface imprinted MIPs on carboxyl functionalised carbon nanotubes. It was established in this paper that the photoluminescence properties of the chelated lanthanide ions increased significantly after complex formation with the surface carboxylic groups. Furthermore, binding of the target protein caused a further increase in fluorescence in a concentration dependent manner over a wide linear concentration range (3.1–150 U mL^{-1}). In addition, the method utilized phosphoserine as a dummy instead of the whole bulky protein, since CA 125 is a large protein (200 kDa) that would not be easy to imprint or remove wholly. Recently, in 2020, a comparable method used the lanthanide metal Tb (III) to develop fluorescent MIPs for detection of melatonin hormone [311]. Owing to the high photostability, negligible auto-fluorescence, lack of photobleaching, and long lifetime offered by lanthanide complexes, we believe the use of lanthanides as superior alternatives to QDs will be the future in the fluorescence sensors field.

4.1.6. Miscellaneous cancer biomarkers

An electrochemical biosensor was developed for malignant pleural mesotheliomas' biomarker; Hyaluronan-linked protein 1 [240]. Optimisation and imprinting studies were performed on BSA, which has a similar size and molecular weight to the target protein. Afterwards, the optimised method was applied for the target protein imprinting and further application. The authors may have used this approach due to the high cost or the extremely low available amount of the target protein. However, we like to point out that such strategy can lack effectiveness, this is because the alternative protein should not only be similar in size and molecular weight but also in conformational folding, surface orientation of functional groups and net charge, since all these factors contribute to imprinting efficiency, they should be taken into account.

As previously mentioned, metal coordination bonding between template and functional monomer can be a very useful solution for imprinting of proteins in aqueous media. This maneuver was applied for epitope imprinting of the cancer biomarker Kininogen and its fragments (K-2209 & K-1944) [241]. Kininogen is an important biomarker that is diagnostic for many cancer types including gastric, liver and colorectal cancers [312]. The authors reported the use of MAA and Am as functional monomers, however the selectivity was not satisfactory. Therefore, metal coordination was used as a suitable alternative to the more commonly employed hydrogen bonds between the template and the monomers. Copper (II) was chosen as the metal coordinator and 4-VP as functional monomer. Cu(II) provided a complexation position with the imidazole groups in the histidine residues of one of the used epitopes and it could also complex with the carboxylic group of the aspartic acid moieties at the C-terminal of the other epitope. Through the careful optimisation of the ratios of template to metal to functional monomer, successful hierarchical imprinting with maximum binding affinity was performed on the surface of a porous silica support. Hierarchical imprinting refers to an approach to confine the binding sites to open entrance and highly accessible domains

of the polymer. This technique is executed through template immobilisation on a porous substrate, mostly porous silica beads like the ones applied in this method followed by polymerisation. Afterwards, silica support is removed by etching and open entrance cavities are created and confined at the surface to allow for binding of large epitope bearing protein targets [313]. However, we would like to point out that these large cavities themselves can also entrap some of the similar structure and small protein fragments found in the sample leading to non-specific binding.

An electrochemical sensor was developed for three different epitopes of regenerating protein 1B, a useful urinary biomarker for diagnosis of pancreatic ductal adenocarcinoma [314]. The first method used surface imprinting of ethylene-co-vinyl alcohols via solvent evaporation in DMSO on a gold screen printed electrode [243]. Later in 2018, the same authors used their method for determination of the target in cell culture [242]. However, due to the complexity of sample, the authors developed magnetic MIPs with the same composition for selective extraction of target from the cell culture. Therefore, there were two MIPs used in this study, one for extraction of target (magnetic) and one for electrochemical sensing. This boosted the selectivity as the target was selectively extracted twice from the sample, which significantly reduced interferences and enhanced sensitivity by pre-concentration prior to analysis.

Vascular endothelial growth factor (VEGF) is a very important protein normally involved in the process of angiogenesis in wound healing as well as some other pathological conditions. However, high level of VEGF is associated with the diagnosis of growth of solid tumors encountered in different cancer types [315]. An “out of the box” fluorescence method was reported for determination of VEGF with an application to tumor cells xenotransplanted in zebrafish embryos [244]. The method used solid phase synthesis for the production of MIPs, then to add the fluorescent component, QDs were covalently attached to the surface of MIPs instead of being embedded in the core. The authors explained the use of this strategy to the fact that excessive quenching was noticed for QDs embedded in MIPs core, therefore, it was a better alternative to covalently attach them to the surface. This was the first publication to come to our knowledge in which QDs were not in the core of the developed MIPs. In this publication, we notice that the emission peak of QDs retained the same original intensity after covalent attachment with only a small peak shift. We believe this strategy allows for a wider concentration range to be determined based on quenching of the native fluorescence. Nonetheless, there is no information on the linear range or the LOD of the method.

MIPs are proposed as a better alternative for antibodies for selective binding of targets, however that does not eliminate the potential use of antibodies along with MIPs to produce more successful results. In the electrochemical method reported by M. Johari-Ahar et al., authors developed a novel method for dual detection and determination of VEGF along with another important cancer biomarker; epidermal growth factor receptor (EGFR) [245]. In this method, MIPs layer was deposited by surface imprinting on a gold screen printed electrode in the presence of the two template proteins to produce cavities complementary to both targets. However, in order to separate the electrochemical signal of each target, liposomal amplification was applied. Liposomes for each target were loaded with metal cations: (Cu^{2+}) for VEGF and (Cd^{2+}) for EGFR and decorated with antibodies specific for each target to enable each liposome to identify its target protein. After selective binding of targets in the sample to the MIPs layer, liposomal solution was added, and each liposome bound selectively with its target protein by means of the attached antibodies. After washing and lysing, the corresponding metal cations were left at the complementary cavities and were determined by potentiometric striping analysis, where each metal induced signal corresponded to the

concentration of each target. There were stability concerns regarding the use of the antibodies, however the authors addressed the stability issues and confirmed that the sensor was stable for a period of 75 days and the liposomal solution was stable for 60 days. Nonetheless, there are concerns regarding the cost effectiveness and the complexity of preparation of the applied solutions.

4.2. Cardiac diseases biomarkers

Troponin I is a vital biomarker for acute heart failure [316]. Among the interesting methods for its determination is an optical sensing SPR method using norepinephrine as functional monomer [256]. The rational for using norepinephrine was based on the structural similarity with dopamine, which is a very commonly used monomer for MIPs based on self-polymerisation. The only structural difference between both neurotransmitters is the presence of the hydroxyl group in the benzylic position of norepinephrine. Therefore, researchers in this study had the idea of trying norepinephrine instead of dopamine and investigating the effect of the extra hydroxyl group on the binding behavior. Through comparison of poly(dopamine) (PDA) and poly(norepinephrine) (PNE) MIPs, it was found that the extra repeated hydroxyl group of PNE increased the wettability of the surface and hence increased the hydrophilicity of the MIPs film. Consequently, there was a reduction in hydrophobic nonspecific binding and enhanced selectivity towards troponin I. Moreover, these findings were confirmed by contact angel measurement and binding studies against other interfering proteins. Nevertheless, the sensitivity of the method was not satisfactory for clinical applications, therefore, a monoclonal antibody was applied to selectivity bind the troponin I subunit, which was not involved in MIPs binding, more like sandwich mode of ELISA assay. This approach caused an amplification of signal and augmented sensitivity significantly. However, the use of antibodies caused stability and cost issues for the method, which MIPs technique was introduced to solve in the first place.

Pyrrole is one of the most commonly used functional monomers in electropolymerisation of biosensors. This is due to its ease of polymerisation at low potential, its compatibility to variety of applications and its high conductivity [317]. In most of the published papers, pyrrole is used alone for electropolymerisation, yet it is useful to consider if other functional groups can amplify the biomimetic properties of the resulting MIPs films. In the developed electrochemical method for determination of cardiac troponin T, another sensitive and specific biomarker for diagnosis of heart failure, the authors considered the use of carboxy-pyrroles as functional monomers along with pyrrole [250]. 2-carboxy pyrrole and 3-carboxy-pyrrole were tested as co-monomers with pyrrole and resulting MIPs were evaluated according to their binding behavior. It was found that 3-carboxy-pyrrole provided a better binding of target as it caused more interactions with the reactive sites of troponin T. However, 2-carboxy-pyrrole was not a suitable co-monomer, since it prevented efficient polymerisation and caused steric hindrance at the binding sites, so 3-carboxy-pyrrole was chosen as the co-monomer. The usefulness of carboxy-pyrrole was attributed to the fact that troponin T existed in protonated form in the electropolymerisation solution, which provided an electrostatic interaction with the negative carboxylic groups and hence increased binding affinity. In addition, researchers conducted an optimisation study on the ratio of pyrrole to 3-carboxy-pyrrole for optimum binding. The optimum ratio was 1:5, which in itself was an indicator as to the important role of 3-carboxy-pyrrole in selective binding of target. This study highlights the importance of investigating functional monomers that can add more types of bonds between target and the binding sites, which in turn can increase sensitivity and reduce nonspecific binding. The use of the mixture

of pyrrole and 3-carboxy-pyrrole was employed later in a very recent paper for determination of cystatin C, a biomarker of renal diseases, by the same authors [291].

Fluorescent MIPs sometimes suffer a background noise effect from the residual unbound MIPs after binding with the target, which limits the application of this method in the sensors field. A possible solution is to remove the unbound fluorescent MIPs from solution after target binding and before fluorescence measurements. M. V. Sullivan et al., introduced a very handy method to remove the unbound MIPs through the use of superparamagnetic iron oxide NPs (SPIONS) [264]. Myoglobin (Myo), one more important cardiac biomarker for ischemia and acute heart failure, was chosen as the target of this method. Fluorescent MIPs were synthesized for Myo using fluorescein O-acrylate as fluorescent functional monomer. Following binding of target in the sample to the developed MIPs, SPIONS were added after the equilibration time to remove the unbound MIPs in a method similar to magnetic fishing. Glutaraldehyde was used as a functionalising agent to bind Myo to SPIONS, then SPIONS bound to Myo were used to attract the unbound MIPs in sample solution to selectively bind with the attached Myo residues. Subsequently, the bound SPIONS-Myo-MIPs were removed by magnet and the fluorescent MIPs were measured with no interference. Moreover, the authors used an innovative, green and time saving method for one-pot solvothermal microwave assisted synthesis of SPIONS in less than 20 minutes. This green and time saving method is an important contribution that can expand the field for the use of SPIONS with more target proteins in future fluorescence methods.

4.3. Neurological diseases biomarkers

Amyloid-beta (Aβ) is a polypeptide involved in neuronal death and formation of brain senile plaques, therefore it is a well-established biomarker of Alzheimer's disease [318]. **Table 1** shows four electrochemical MIPs methods for determination of Aβ; all four methods are based on electropolymerisation of functional monomer in presence of the template protein. However, we noticed an obvious difference in the sensitivity of these methods as evidenced by their LOD values and, this difference can be attributed to the working electrode used in each method. In the first method [269], the authors used α-cyclodextrins (α-CDs) as functional monomers; this was very beneficial, since α-CDs could incorporate the target protein in their core without any conformational changes thus maintaining target integrity comparable to that found naturally. However, poly(α-CDs) is a non-conductive polymer, therefore the working electrode needed to be modified with a conductive layer prior to electropolymerisation. Herein, authors used aniline to form an electropolymerised layer on the working electrode underneath the poly(α-CDs) layer. The poly(aniline) layer increased conductivity, allowing for more efficient polymerisation of α-CDs, and enhanced target chemical recognition by promoting fast electron transfer between the negatively charged target and the positively charged poly(aniline) layer. Nonetheless, this method showed the least sensitivity among the four methods. The second method applied electropolymerisation of o-phenylenediamine on a paper based carbon ink electrode [271]. In addition to the previously discussed advantages of paper strip sensors, paper sheets have a porous and rough structure of fibres that can be easily modified. Carbon ink was chosen as a conductive electrode, since it is cheap, chemically stable and conductive material. However, to increase conductivity an imprinted layer of (EDOT) was added to the carbon ink electrode before molecular imprinting. PolyEDOT layer is a highly conductive polymeric layer and it also increases surface roughness and therefore increases the number of reactive sites per unit area. Consequently, we can see an enhancement to the level of sensitivity by almost three times.

Increase in surface area contributes significantly to conductivity, therefore, using modifications to the working electrode that can increase surface area will improve sensitivity. The third paper for determination of Aβ used electropolymerisation of aniline on carbon electrode modified with copper NPs doped carbon nanotubes (CNTs) [270]. It is worth mentioning that most electrochemical methods employed in sensors are indirect methods, which depend on the use of redox probe to check alterations in current upon template interaction. The remarkable innovation of this method was the use of electroactive metal species doped in the working electrode (copper NPs), which eliminated the need for redox probe and enabled simpler mode of operation. In this method, we also observe the use of CNTs, which provided high conductivity, chemical stability, increased surface area and amplified mass transport. In return, we notice an increase in sensitivity compared to the previous two methods, which proved the critical role of the electrocatalytic area of the working electrode. Finally, the fourth and most recent method for Aβ determination published in 2020 showed an unprecedented level of sensitivity with LOD of 0.3 fg mL^{-1} [319]. This very low LOD was attributed to the modification of the working electrode with MXenes and MWCNTs in the ratio 3:1. Unlike the single walled carbon nanotubes used in the second method, MWCNTs consist of multiples layers of graphene in a concentric pattern, which increases the exposed surface area dramatically besides being a highly conductive material. On the other hand, MXenes are 2-dimensional early transition metal carbides, and in this paper, titanium carbide was used. The merits of MXenes such as high conductivity, large surface area, ease of fabrication and compatibility with organic or aqueous phases, combined with the perks of MWCNTs and the use of pyrrole as functional monomer, all gave a very effective electrochemical sensor with very high sensitivity.

Eventually, the overview of these papers for determination of Aβ highlights the importance of a working electrode modification step. Modifications should be aimed to increase not only conductivity, but also to increase the total surface area, which in turn can promote the performance of the method.

4.4. Plasma Iron levels biomarker

Transferrin (Trf) is an approximately 80 kDa glycoprotein molecule normally found in the human body in the concentration range 204-360 mg dL^{-1}. The main roles of Trf as biomarker are to assess iron levels in the blood and to diagnose iron related disorders. Moreover, it can also be biomarker for neurological diseases [320]. Among the analytical methods we like to discuss, a plasmonic SERS method based on self-polymerisation of dopamine that caught our attention. The method uses gold nano-rods as substrates with a comparison of how their surface arrangement as stacks or aggregates or self-assemblies could influence the method [274]. However, we noticed that dopamine was polymerised in Tris buffer of pH 7.5, this pH is lower than the required pH for self-polymerisation of dopamine, which is known to polymerise only at pH above 8 usually 8.5 [321]. The authors did not give an explanation for the choice of this pH value, nor did they explain how polymerisation proceeded at this pH. Moving on to a later paper published in 2018, magnetic and fluorescent MIPs were developed for Trf through post-imprinting modification [322]. Core shell surface imprinting was performed on the surface of the magnetite NPs using 3-carboxyphenylboronic acid as functional monomer. This monomer was very useful, since it formed a bond between the carboxylic group and the Fe ion on surface of magnetite enabling monomer molecules to be anchored to the surface. In addition, boronic acid affinity to cis-diol moieties found in Trf enabled a reversible covalent bond between template and the binding sites of MIPs. Following imprinting and template removal, a semi-cyanine fluorescent compound was adsorbed on the surface

of MIPs to impart the fluorescent property that was quenched by target binding. However, fluorescence quenching was also noticed for NIPs to a lower extent. The authors attributed this phenomenon to the electrostatic interaction between the electron rich target molecules at pH 7.4 and the N^+ moieties in the fluorescent molecules leading to a shift in the electron density on the fluorescent molecules and subsequent quenching. Although this limit of quenching seems insignificant, it is still problematic especially at high concentrations of Trf where the electrostatic interaction is expected to increase. In this case, there are two factors affecting quenching not only target binding, but also the target electrostatic interaction with the fluorescent molecules on the surface.

The final study that we like to highlight concerning Trf as target is an electrochemical method that is more focused on studying the interaction between targets and the transducer surface. It is generally assumed that the binding of target happens exclusively at the MIPs layer surface, however in this study, the authors focused on the possible interaction between target or other cross reacting proteins with the bare transducer surface [276]. This paper presented some interesting findings that need to be taken into account when conducting electrochemical or SPR methods. The first finding was that the adsorption of target or the other cross reacting proteins suppressed the bare electrode signal and, this proved the validity of target pre-adsorption on the electrode prior to electropolymerisation. The second finding is the effect of the template removing agent. Herein, the authors used enzymatic degradation via proteinase K, therefore, the effect of proteinase K on the bare electrode was investigated and it was found to suppress the redox signal as well. This suggests that any remaining enzyme as well as the resulting protein fragments after washing can cause significant reduction in redox signal. Consequently, the electrode needs to be washed thoroughly after enzymatic degradation until the signal reaches a constant value. The third interesting finding was the cross reactivity with similar proteins, which might be considered as defect in the method's selectivity. However, it highlighted the importance of studying binding on shape, size and electrostatic interaction basis. Researchers showed a cross reactivity with iron-free apo-Trf in MIPs developed via template adsorption compared to MIPs prepared by prepolymerisation mixture approach. This was due to the fact that protein adsorption to the bare electrode surface can cause protein unfolding and therefore cause non-specific binding with similar proteins. On the other hand, there was no considerable cross binding with the protein ferritin, this could be explained by the existence of electrostatic repulsion between ferritin and scopoletin functional monomer at low ionic strength. Urease was the one cross reacting protein with maximum non-specific binding. The authors explained this to happen due to the shape of the urease resembling a hammer head that could access MIPs cavities, in addition to the presence of many cysteine moieties in urease structure capable of strongly interacting with the bare gold surface. Therefore, it is important to always conduct a study on the effect of the transducer substrate and its possible interactions with the template itself, cross reacting proteins or template removing solutions in any electrochemical-MIPs method.

4.5. Miscellaneous biomarkers

Pepsin enzyme is protein in nature with approximate molecular weight of 35 kDa [323]. Recently, it has been shown in some studies that salivary pepsin can be used as a potential biomarker for different gastric ulcers [324]. One method for detection and quantitation of pepsin uses magnetic MIPs in an ELISA format while applying fluorescent polystyrene beads (PSB) as labels. The principle of the method depended on immobilisation of the developed magnetic MIPs to the walls of the wells of a microtitre plate via magnetic inserts. The sample solution containing pepsin was mixed with PSB and subsequently pepsin was adsorbed onto

their surface by hydrophobic interactions. Afterwards, this solution was added to the wells and pepsin with the adsorbed PSB bound with the magnetic MIPs resulting in a corresponding decrease in central fluorescence of the solution. The advantages of this method included the use of MIPs instead of the conventional antibodies and the use of PSB instead of the enzyme label. Moreover, there was no need for a washing step after the addition of sample solution. Nonetheless, this method suffered from various drawbacks that needed to be addressed. Firstly, the authors mentioned that very high concentration of target protein could saturate MIPs binding sites as well as PSB leading to a reduced amount of PSB drawn to the magnetic inserts. Therefore, it was necessary to carry a binding kinetics study to determine the maximum concentration of pepsin that MIPs could bind to avoid such problem, yet this study was not presented. Secondly, MIPs synthesis utilized three different functional monomers, which can increase non-specific interactions and binding sites heterogeneity through side reactions, yet the rationale for using three different monomers was not explained, nor why they used them in these ratios. Thirdly, an elucidation of the functional groups of pepsin involved in the binding with MIPs or PSB should have been included to verify that hydrophobic binding with PSB did not adversely affect binding with MIPs. In addition, if PSB caused conformational changes to the structure of pepsin, this might result in total loss of binding selectivity. Finally, authors mentioned they switched blue PSB to red PSB when applying the method to synthetic gastric fluid due to the effect of hydrochloric acid on binding with blue PSB and reduced sensitivity. However, the question is why did they not use red PSB from the start, especially given that all of the optimisation studies were carried out using blue PSB. Despite the promising approach used in this method, it lacked some fundamental elucidations and experiments to verify its usefulness.

A very similar method to that used for pepsin was applied for determination of insulin and leukotriene (LTE4) biomarkers. The method also used a modified microtitre well plate with magnetic inserts and magnetic MIPs/ magnetic NPs [288]. However, the authors of this paper did not use PSB as the fluorescent label, instead they used fluorescent nanoMIPs imprinted for the target proteins. In the case of LTE4 (as a small molecule), we have three elements involved in the assay: fluorescent nanoMIPs, LTE4 sample, and magnetic NPs with immobilised LTE4. The assay here depends on competition between free LTE4 in sample and immobilised LTE4 on the binding sites of the fluorescent nanoMIPs, where the change in fluorescence corresponded to the concentration of target. In the case of insulin (as a large molecule), we similarly have three elements: fluorescent nanoMIPs, magnetic MIPs and the insulin sample. The assay in this case was in sandwich format. The magnetic MIPs were added first to the well and immobilised via the magnetic inserts, followed by the addition of target and fluorescent nanoMIPs. Subsequently, both MIPs bound to insulin molecule on opposite sides creating a stable complex in which insulin was sandwiched between the two MIPs. This led to a decrease in fluorescence intensity, corresponding to the concentration of insulin. It is important to mention that most of the flaws of the first method using pepsin as target were avoided in this paper. Authors conducted a full computational study on the structure of the target and possible interactions with different functional monomers, no PSB were used, and a complete characterisation profile of the produced MIPs was presented.

It is always useful to combine new, simple, sensitive and cost-effective analytical methods with the technique of molecular imprinting. In the following method, the authors presented a novel combination between photonic crystal sensor and MIPs for determination of the venous thromboembolism biomarker fibrinopeptide B [289]. Photonic crystals consist of periodic materials arranged in a way to prevent certain wavelengths of light passing through, therefore

they can be synthesized in various coloured structures and different reflected wavelengths. Moreover, when these crystals are coupled to biorecognition elements such as MIPs, changes to the conformation of polymer upon binding of target can induce changes in light reflectance enabling label free optical transduction. In their work, MIPs were surface imprinted on a colloidal array of a monodispersed silica NPs sensing layer. The prepolymerisation mixture was squeezed in the small cavities in the silica NPs crystal via sandwiching between two glass slides in a way similar to soft lithography. The resulting MIPs were capable of highly sensitive determination of the target with corresponding change in reflectance spectra in a concentration dependent manner. In addition, to ensure that the change in reflectance was due to the binding of target only, NIPs were investigated, and it was shown they had negligible effect on the reflectance spectra. Further control experimentation was carried out to inspect binding of fibrinopeptide B target with bare photonic crystal and, it was found that in the absence of MIPs the target had no effect on peak intensity. This control study confers with the previous recommendation we reviewed to study the influence of bare substrate interaction with target and competitive proteins [276].

5. Recommendations

We would like to present some recommendations based on our review to assist in guiding the wheel of MIPs research to the right direction.

- It is very important to carry out a complete binding studies profile. Within our survey, we found a number of papers missing some or all the binding studies (binding kinetics, binding isotherm and binding selectivity). Binding studies verify the applicability of the method and are a standard for their effective synthesis. Comparison to NIPs in terms of binding affinity should be presented in the optimisation graphs and binding tests results to indicate the higher selectivity and usefulness of MIPs. Moreover, some of the selectivity studies were performed on irrelevant proteins; it is important when researchers conduct a cross-reactivity study to choose analytes that can possibly coexist with the target in the same sample and could cause real errors in analysis.

- The washing step of the developed MIPs to remove the template molecules after polymerisation is a very significant and important step that can affect the successful application of MIPs. Washing solutions vary between concentrated salt solution, mixtures of acetic acid and sodium dodecyl sulphate, proteinase enzymes, organic acids or buffers. The choice of washing solution cannot be just arbitrary or based on previously published papers for similar analytes. As a matter of fact, the choice of washing solution should depend on specific optimisation to verify complete template removal and to ensure that MIPs binding sites are not compromised by any harsh conditions. An optimisation process for the washing step that includes type and concentration of washing solution and number of washing cycles should be presented. This step is not just routine work; complete washing prevents the problem of template bleeding and increases the availability of binding sites.

- Choice of monomers is the success determining step of MIPs synthesis. The rationale of choosing a particular monomer or mixture of monomers should be based on structure elucidation of the target and its possible interactions with the monomer. Computational design can help save time and energy in conducting many possible interactions between target and monomers and can calculate the corresponding Gibbs energy. Moreover, the chosen monomers can also impart certain desirable properties

to the developed MIPs that may be helpful, such as reduction of nonspecific binding and increased hydrophilicity. As a result, we strongly recommend that researchers explain in their papers the reasons behind the choice of functional monomers.

- Fluorescent MIPs based on core shell imprinting on fluorescent substrates are applied in a significant number of papers. However, in case of application of MIPs to the human body, a toxicity profile of the fluorescent substrates should be taken into account. Quantum dots are usually manufactured from heavy metals such as cadmium and tellurium that can cause heavy metal poisoning and cytotoxicity. Therefore, safer alternatives should be considered in this case such as carbon dots. Carbon dots are less toxic, more environment friendly than quantum dots, and they have higher photo stability and chemical inertness. We also would like to point to the problem of fluorescence background from unbound fluorescent MIPs upon application to target sample. A simple and innovative approach to solve this problem was presented through the use of SPIONS, as discussed above [264].

- Electropolymerisation of monomers on the surface of substrate/electrode is a very widely applied and easy method for polymerisation. However, there should be a careful control over the concentration of monomer solution and, electrochemical scan rate as well as the number of scan cycles, since they control the thickness of the resulting polymer film. Very thin films can only bind a small part of the target molecule resulting in reduced sensitivity. On the other hand, very thick films can cause permanent enclosure of the template molecules within the polymer matrix and consequent loss of activity [80].

- We strongly advise to conduct a stability study on the developed sensors for bioanalytical applications, such as a repeated assay using the sensor over time intervals to show if the performance of the sensor remains the same or deteriorates. Based on this study, researchers can establish a time frame in which the sensor results are considered reliable and reproducible.

- pH is a crucial variable in MIPs synthesis especially for proteins. Different pHs can result in different conformations of the same protein molecule. If MIPs are imprinted on a conformation of protein that is different from its physiological conformation, that basically can terminate the applicability of the method. Therefore, it is recommended to carry out synthesis in near physiological pH value (7.4) and in a hydrophilic environment comparable to that of body fluids to increase applicability and sensitivity.

6. Conclusion

Molecularly imprinted polymers technology is a fast-growing trend in analytical chemistry as well as in other fields of science. MIPs greatest contributions are specifically in the field of sensing and biosensing, this is due to their ease of synthesis, cheap starting materials, high selectivity, long term stability, and many other merits. There are multiple methods of synthesis of MIPs, multiple starting materials and many characterisation methods that are explained extensively in this review. However, the choice of which kind of MIPs to apply for determination of a certain analyte depends mainly on the type of analyte itself. Moreover, it is of prime importance to carry out a thorough optimisation of the synthesis procedure covering all elements involved in synthesis as well as all elements influencing the polymerisation reaction such as ionic strength, pH and temperature. Following synthesis of MIPs, there should be a complete characterisation profile (physical, thermal, chemical and

functional) for the resulting MIPs to ensure their usefulness for their intended application. Furthermore, in this review, we focus on the application of MIPs technology in the field of biomarkers' analysis. Protein biomarkers are among the most abundant biomarkers in the human body; therefore, they were chosen as the target of our review. Numerous papers were collected in our literature survey in the well-known scientific databases for the ten years' period from 2011 to 2021. The collected papers were meticulously reviewed and summarised in table form showcasing their important aspects. In addition, a critical review of some of the surveyed papers is presented. In this critical review we try to direct a spotlight on the most novel ideas, recent improvements in the field of MIPs, successful collaboration between MIPs and other analytical or sensing methods. Nonetheless, we also point out to possible errors in few methods, such as missing studies or experiments as well as some contradictions. The ultimate goal of this review, is to start a scientific constructive debate, promote future research in the field, and to help researchers avoid some of the common mistakes.

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Figure captions:

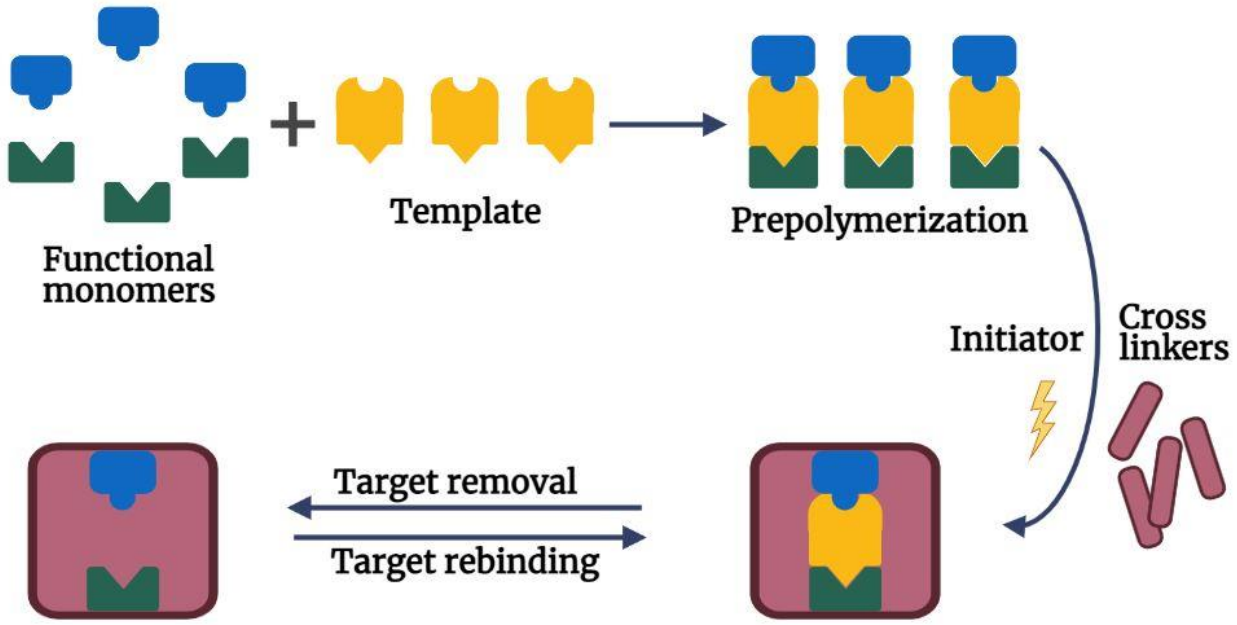
Figure 1: Schematic illustration of the process of molecular imprinting and target removal/rebinding.

Figure2: Chemical structures of common a) functional monomers b) cross linkers c) azo initiators.

Figure3: Schematic illustration of the process of core shell imprinting on the surface of nanoparticles.

Figure 4: Schematic illustration of solid phase synthesis of nanoMIPs. Prepolymerisation mixture is added to the column containing glass beads with immobilised template followed by initiation. Unreacted monomers and low affinity oligomers are eluted at low temperature. NanoMIPs are then eluted at higher temperature.

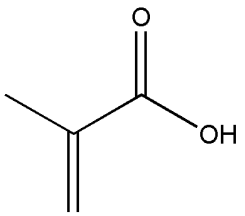
Figure 5: Cartoon representation of the possible origins of binding sites heterogeneity during molecular imprinting. a) Different species in prepolymerisation mixture showing different possible complexes between template and monomer molecules including 1:1, 1:2 and 1:3 ratios in addition to free monomers. b) Structure after polymerisation showing heterogenous binding sites due to different binding sites accessibility and various polymer backbone conformations. c) Structure after polymer collapsing and fracturing due to template removal, solvent exchange and polymer swelling.


Functional monomers
Template
Prepolymerization
Initiator
Cross linkers
Target removal
Target rebinding

1

Figure 1

Figure 2



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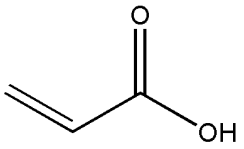

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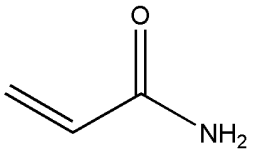

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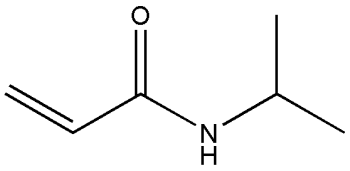

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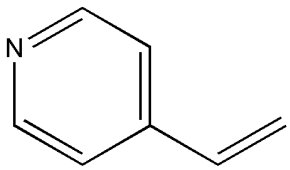

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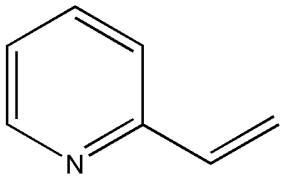
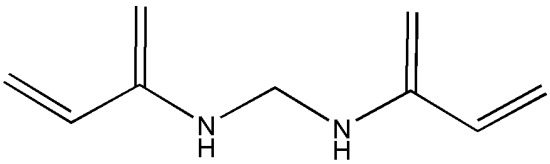


2-VP

Figure 2b



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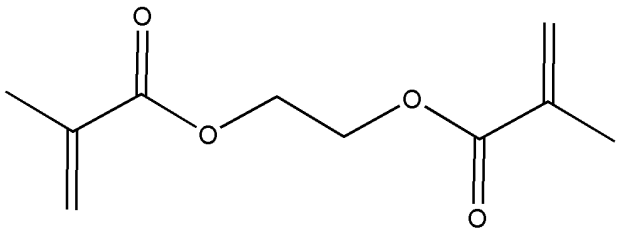

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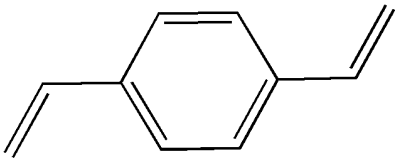
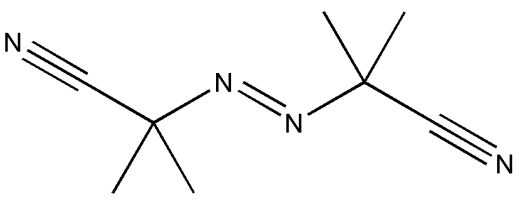


DVB

Figure 2c



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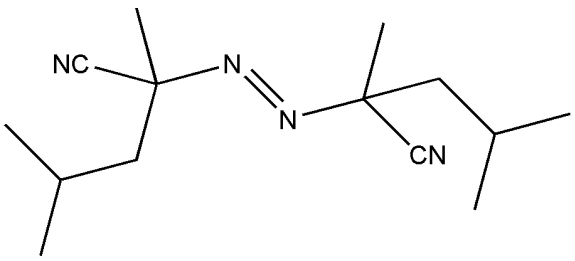

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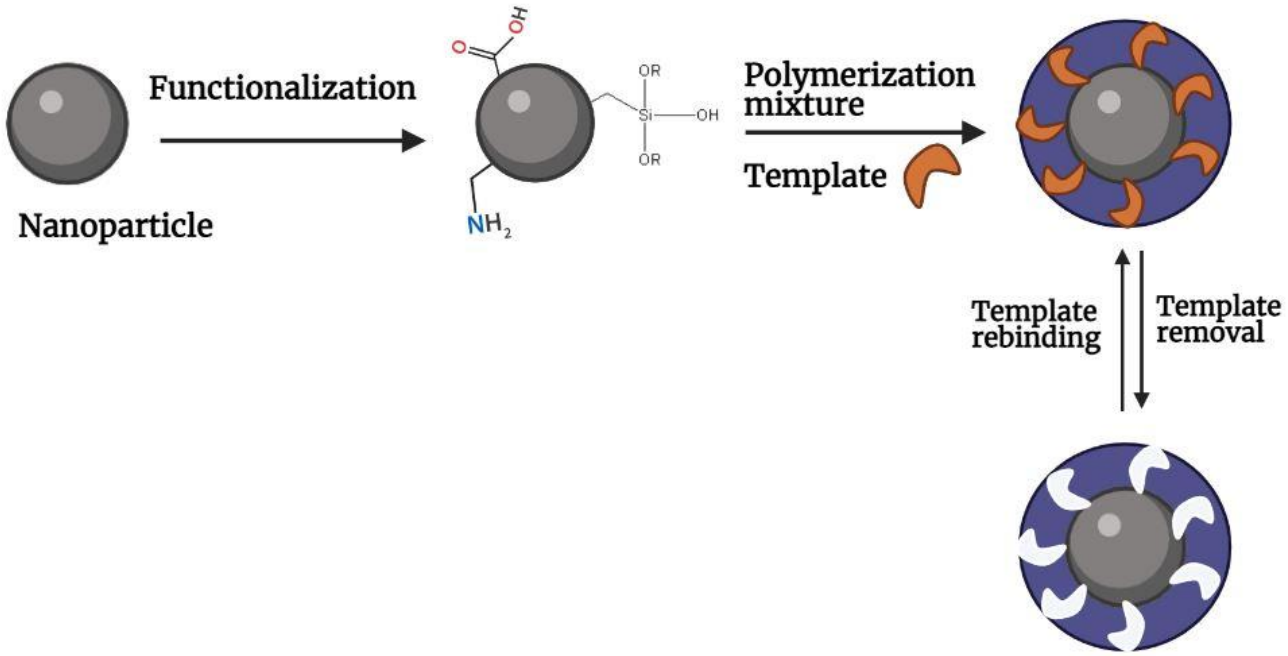


Figure 3

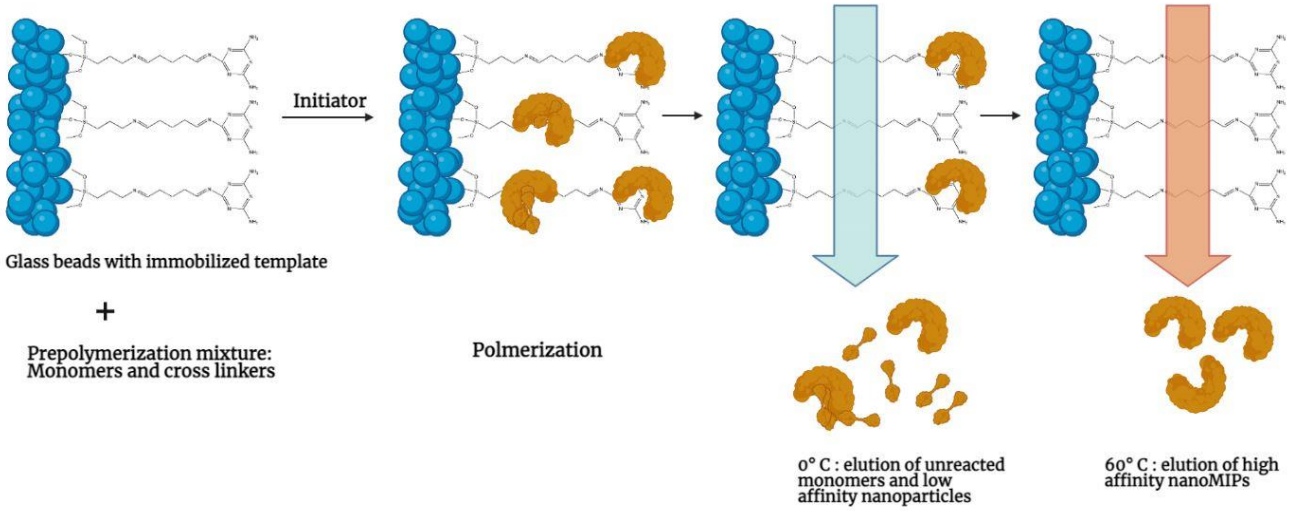


Figure 4

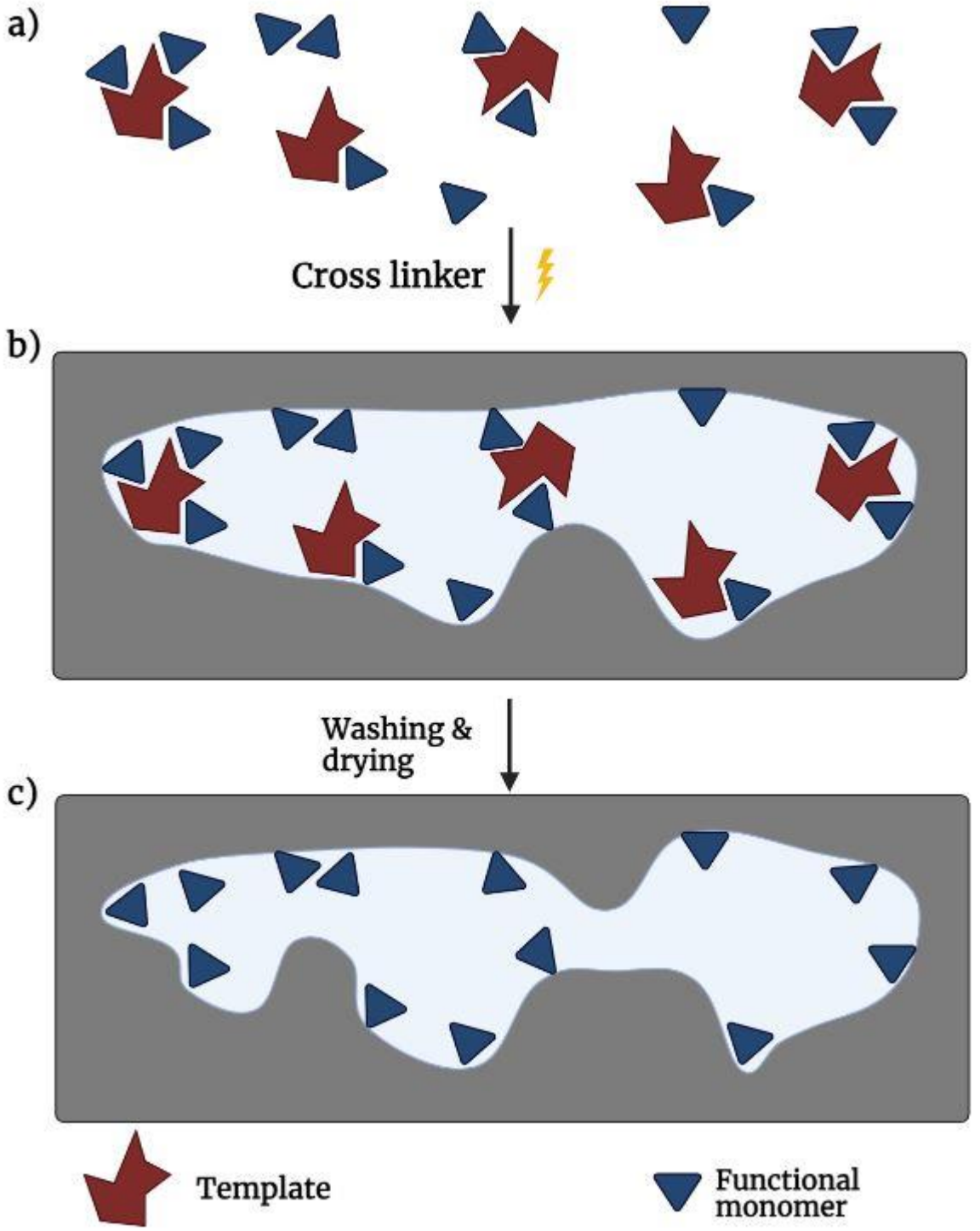
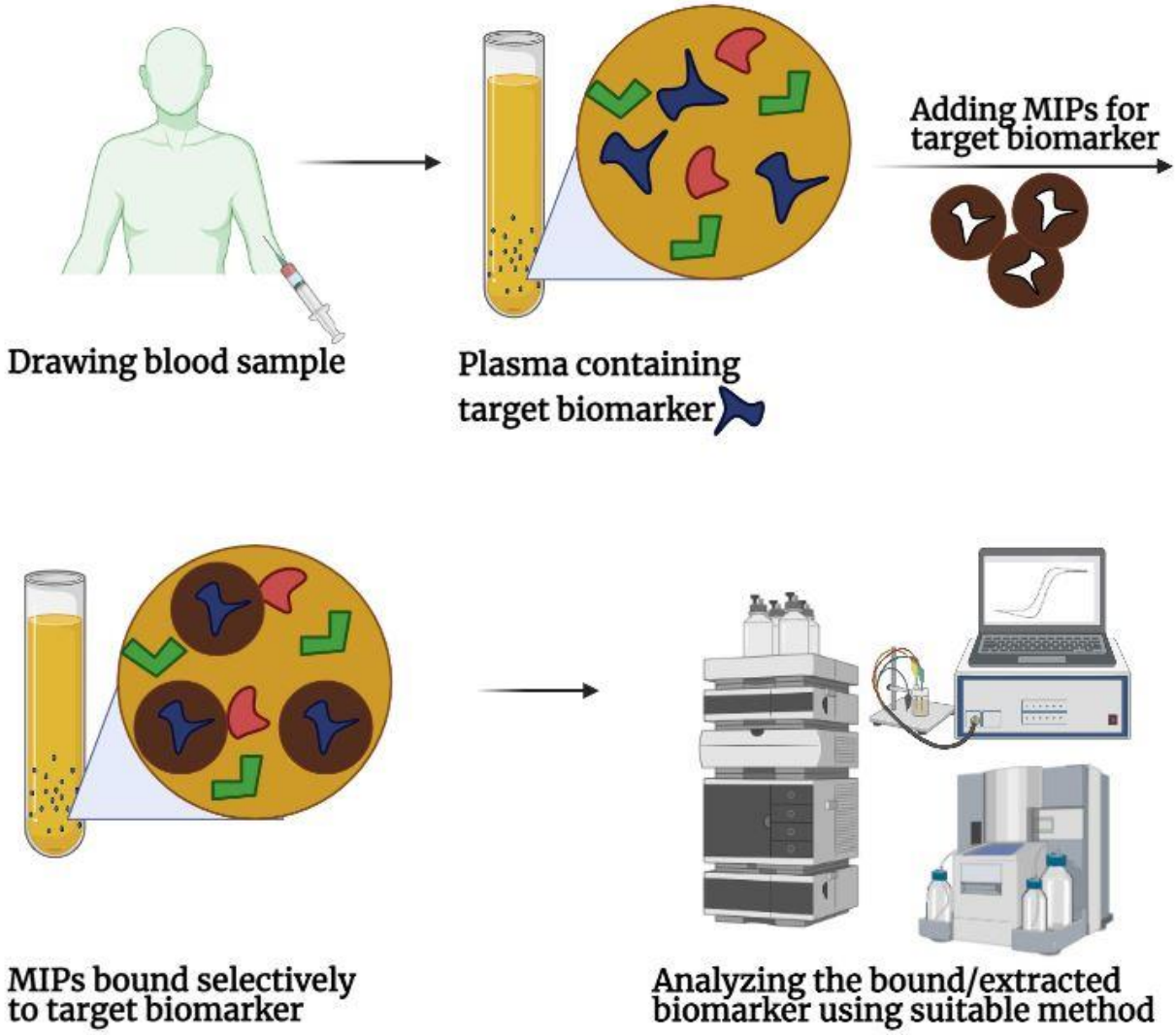

a)
Cross linker
b)
Washing &
drying
c)
Template
Functional
monomer

Figure 5



Graphical abstract