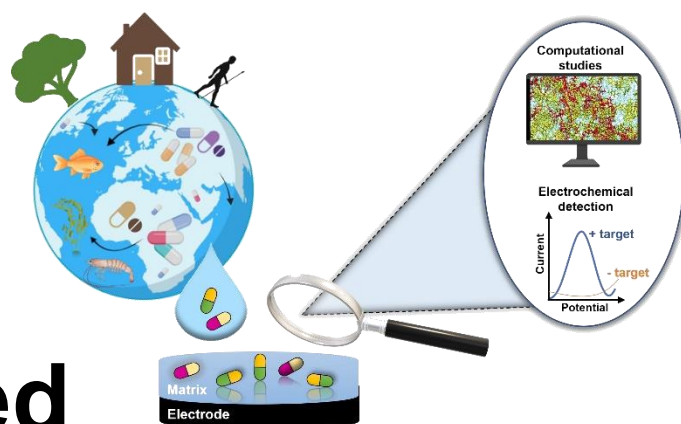


Computer-aided design of molecular imprinted electrochemical sensors for analysis of pharmaceuticals in waters



Patrícia Catarina Santos Rebelo

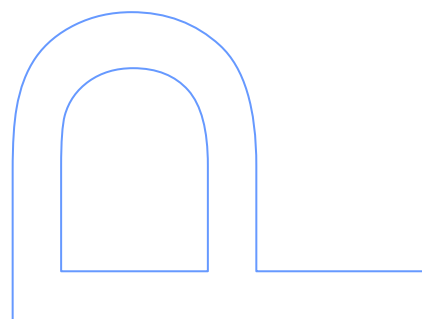
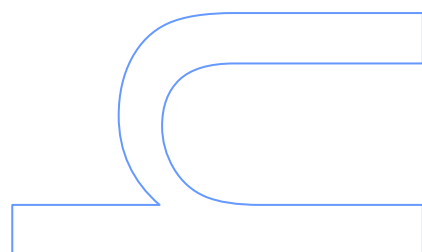
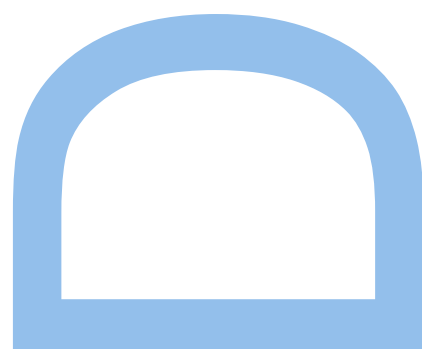
Doutoramento em Química Sustentável
Departamento de Química e Bioquímica
2022

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Computer-aided design of molecular imprinted electrochemical sensors for analysis of pharmaceuticals in waters

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A dissertation presented to the Faculty of Science from Porto University, Nova School of Science and Technology from Lisboa and Aveiro University for the degree of Doctor in Sustainable Chemistry



Porto, 2022

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This work was supported by the Associate Laboratory for Green Chemistry – LAQV, which is financed by national funds from “Fundação para a Ciência e a Tecnologia (FCT)/Ministério da Ciência e Ensino Superior (MCTES)” [UIDB/50006/2020, UIDP/50006/2020]. The work was further supported by the projects PaperSenseMIP (PTDC/QUI-QAN/3899/202) and FARMASENSE (NORTE-01-0247-FEDER-039957) sponsored by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, by Programa Operacional do Norte (NORTE 2020) and Fundo Europeu de Desenvolvimento Regional (FEDER). This research was also funded by the EU and FCT/UEFISCDI/FORMAS, in the frame of the collaborative international consortium REWATER-Sustainable and safe water management in agriculture: increasing the efficiency of water reuse for crop growth while protecting ecosystems, services and citizens’ welfare (WaterJPI/0007/2016), which was financed under the ERA-NET Cofund WaterWorks2015 Call, as an integral part of the 2016 Joint Activities developed by the Water Challenges for a Changing World Joint Program Initiative (Water JPI) and through the Biodiversa & Water JPI joint call for research proposals, under the BiodivRestore ERA-Net COFUND programme DivRestore/0002/2020, and cofunding by FCT, Portugal. Patrícia Rebelo is grateful to FCT and European Union (EU) for the financial support through the doctoral research Grant SFRH/BD/132384/2017 and exceptional Grant COVID/BD/151963/2022, financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu (FSE) and MCTES.



The studies presented in this thesis were performed in “Grupo de Reação e Análises Químicas – GRAQ” (member of the Associate Laboratory – REQUIMTE, Laboratório Associado para a Química Verde), at the “Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto (ISEP/PP)” in collaboration with the Department of Chemistry and Biochemistry of Faculty of Science from University of Porto.

*Ao meu Pai
e eterno ídolo*

Agradecimentos

Tenho uma enorme dívida de gratidão para com muitas pessoas que diretamente ou indiretamente tornaram este projeto possível e que me acompanharam desde o início. “Sozinho quase ninguém é quase nada”. Foram 4 anos vividos de uma forma bastante intensa, mas acima de tudo, foram 4 anos muito felizes.

Doutora Cristina Delerue-Matos, minha orientadora, toda a minha gratidão por me ter aberto as portas do GRAQ e por acreditar que era possível ir mais longe. Agradeço todo o seu apoio, compreensão e disponibilidade para ouvir atentamente as dúvidas e desafios que apareceram ao longo do caminho. É um exemplo e inspira-nos a ser melhores pessoas e profissionais.

Doutora Natália Cordeiro, minha co-orientadora, agradeço todo o acompanhamento na parte computacional, a sua disponibilidade e toda a sua prontidão para ajudar sempre que necessário.

Doutor João Pacheco, meu co-orientador, o meu muito obrigada pela oportunidade que me proporcionou ao integrar-me no mundo dos MIPs. Agradeço todo o tempo que dedicou na realização deste projeto e todo o suporte científico.

Doutora Iuliia Voroshylova, por onde começar? Foi uma surpresa agradável e muito enriquecedor poder trabalhar com alguém tão profissional e positiva. Aprendi muito. Agradeço imensamente por me teres recebido de braços abertos e por todo o incansável e essencial apoio que me deste ao longo deste percurso. Admiro a dedicação e sentido prático que colocas em tudo aquilo que fazes.

Doutor André Melo, obrigada por toda a sua disponibilidade e colaboração que foram indispensáveis para o rumo inicial do trabalho.

Doutor Henri Nouws, não poderia não deixar o meu agradecimento por toda a alegria e espontaneidade que sempre trouxe ao laboratório, pelo seu incentivo e por ter sido um grande suporte.

Paulinha, sou grata pela sua bondade e por toda a ajuda que me deu sempre que a si recorri.

Ricarda, uma amiga que deu cor a estes 4 anos e uma pessoa muito especial com quem tive o privilégio de conviver. Obrigada pelos maravilhosos momentos com longas

conversas e confidências, por estares sempre presente e por seres uma animação. A tua amizade torna os meus dias mais felizes.

A todos os meus colegas e amigos do GRAQ, em especial à Diana Pinto e à Isabel, pela companhia, pelos animados almoços, pelos sorrisos e pelas memórias.

Por último, mas em primeiro lugar no meu coração, a minha família e amigos. Em particular:

Mãe, mulher forte e corajosa, sem ti nada disto seria possível! A minha eterna gratidão por todos os valores e princípios que tão bem me soubeste transmitir, pelo teu amor, apoio incondicional e por acreditares em mim. Obrigada pela mãe maravilhosa que és e sempre foste. Tudo isto só tem significado porque tu existes.

Irmão, longe mas sempre presente, obrigada por torceres por mim e por me dares o afilhado mais lindo.

Tia Zeza e a sua linda família, nunca esquecerei o seu apoio. Foram e são pessoas maravilhosas que acreditaram mais nas minhas capacidades do que eu própria. Foram muito importantes neste percurso e fizeram-me sentir sempre o vosso carinho. Obrigada!

Barreto, a minha cara-metade e companheiro de todas as horas, um apoiante dedicado e perspicaz. Não existem palavras que possam descrever o meu orgulho em ti e o meu agradecimento por todo o amor e paciência. Muitos dos valores de que aprecio estão presentes na minha vida graças a ti e é uma honra poder dividi-la contigo.

“Vê-se melhor quando não se vai para ver nada, quando os olhos procuram tudo o que possam achar. E encontram tudo”

(Miguel Esteves Cardoso)

Abstract

Water moves the world and it is imperative for life. Over the years, this precious resource has been increasingly threatened by human action in many ways. Unconscientiously, human activities have been driving to water depletion and loss of its quality. In this direction, there is a growing concern about the occurrence of emerging contaminants (ECs), with special focus on pharmaceuticals, in different water bodies across the globe. The widespread environmental presence of pharmaceuticals has been strongly associated with the inability of Wastewater Treatment Plants (WWTPs) fully degrade these compounds.

Despite efforts of European (EU) water policy well depicted in the development of a Watch List mechanism under EU Water Framework Directives, pharmaceuticals still remain unregulated and the impact of many of them in human and wildlife health is unknown. Their monitorisation and knowledge about respective concentrations in environmental waters is a priority, where High Performance Liquid Chromatography (HPLC) in tandem with mass spectrometry has played an important role. However, following the Principles of Green Chemistry and environmental and economic sustainability, electrochemical sensors have emerged as interesting alternatives owing to their ability to simplify all analytical procedure, where portability and miniaturisation can be easily achieved.

Accordingly, five electrochemical devices based on molecularly imprinted polymers (MIPs), as recognition element, have been developed for the detection and quantification of azithromycin (AZY), furazolidone (FZD), amitriptyline (AMT), atorvastatin (ATV) and citalopram (CTL) in water samples. These pharmaceuticals were selected considering their widespread use and persistence in environmental waters in line with the lack of literature sources reporting the application of electrochemical MIP sensors to detect these specific and important pharmaceuticals.

The sensors were constructed based on the modification of screen-printed carbon electrodes (SPCEs) and carbon paste electrodes (CPEs) surface through electropolymerisation or attached microparticles obtained after traditional precipitation polymerisation techniques. Quantum mechanical (QM) and/or molecular dynamics (MD) computational studies were employed to allow rational selection of an appropriate functional monomer and to simulate the best pre-polymerisation conditions, as well as to understand the prevalent molecular interactions occurring during imprinting process. To improve the efficiency of sensing performance, nanomaterials, namely single- and multi-walled carbon nanotubes (SWCNTs, MWCNTs), were adopted as enhancers and

conductive pathways. Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and scanning electron microscopy (SEM) techniques were applied to characterise the modified electrode surfaces. On the other hand, differential pulse voltammetry (DPV) and square wave voltammetry (SWV) were carried out to directly quantify the current resulting from the oxidation/reduction of the pharmaceuticals after binding in the MIP. Related to the development of the MIP sensor for electrochemical detection of AMT, the mechanism of detection was based on the system tris(2,2'-bipyridyl)ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$)/AMT reaction.

The developed sensors showed good analytical performances and provided sensitive detection with excellent selectivity to recognise the target analyte when compared with other close analogue structures, which can be possible interferents. Additionally, they were successfully applied in spiked river water, tap water or spring water samples, thus demonstrating their potential as a useful and sustainable strategy for monitorisation and quantification of pharmaceuticals.

Keywords: Electrochemical sensors, molecularly imprinted polymers, rational design, computational simulation, molecular modelling, green chemistry, environmental analysis, water.

Resumo

A água move o mundo e é essencial à vida. Ao longo dos anos, este recurso precioso tem sido constantemente ameaçado pelo Homem através de diferentes formas. Inconscientemente, as atividades dos humanos têm conduzido a um esgotamento da água e à perda da sua qualidade. Neste sentido, existe uma preocupação crescente com a ocorrência de contaminantes emergentes, especialmente focada nos fármacos, em diferentes corpos de água em todo o mundo. A presença ambiental generalizada de fármacos tem sido fortemente associada à incapacidade das Estações de Tratamento de Águas Residuais (ETARs) degradarem totalmente estes compostos.

Apesar dos esforços da política hídrica europeia (UE) bem retratada no desenvolvimento de uma Lista de Vigilância sob as Diretivas do Quadro da Água da UE, os fármacos ainda permanecem sem regulamentação e o impacto de muitos deles na saúde humana e na vida selvagem é desconhecido. A sua monitorização e conhecimento das respetivas concentrações em águas ambientais é uma prioridade, onde a cromatografia líquida de alta eficiência acoplada a um detetor de massa tem desempenhado um papel importante. No entanto, seguindo os Princípios da Química Verde e da Sustentabilidade ambiental e económica, os sensores eletroquímicos têm surgido como alternativas interessantes devido à sua capacidade de simplificar todo o processo analítico, onde a portabilidade e a miniaturização podem ser facilmente alcançadas.

Neste contexto, foram desenvolvidos cinco dispositivos eletroquímicos baseados em polímeros molecularmente impressos (MIPs, do inglês *molecularly imprinted polymers*), como elementos de reconhecimento, para a deteção e quantificação da azitromicina (AZY), furazolidona (FZD), amitriptilina (AMT), atorvastatina (ATV) e citalopram (CTL) em amostras de água. Estes fármacos foram selecionados tendo em conta o seu uso generalizado e a sua persistência em águas ambientais em consonância com a falta de fontes na literatura, que reportem a aplicação de MIPs na construção de sensores eletroquímicos para detetar especificamente estes fármacos.

Os sensores foram construídos com base na modificação das superfícies de eléctrodos de carbono serigrafados (SPCEs) e eléctrodos de pasta de carbono (CPEs) através da técnica de eletropolimerização ou através da imobilização de micropartículas obtidas após a polimerização tradicional por precipitação. Estudos computacionais de mecânica quântica (QM) e/ou dinâmica molecular (MD) foram realizados para permitir a seleção de forma mais racional de um monómero funcional apropriado e simular as

melhores condições de pré-polimerização, bem como para compreender as interações moleculares que ocorrem durante o processo de impressão. No sentido de melhorar a *performance* analítica, nanomateriais, nomeadamente, nanotubos de carbono de parede simples (SWCNTs) e dupla (MWCNTs) foram tidos como um elemento potenciador da condutividade elétrica. As técnicas de voltametria cíclica (CV), de espectroscopia de impedância eletroquímica (EIS) e microscopia eletrónica de varredura (SEM) foram aplicadas para caracterizar a superfície modificada dos elétrodos. Por outro lado, a voltametria de pulso diferencial (DPV) e voltametria de onda quadrada (SWV) foram empregues para quantificar diretamente a corrente resultante da oxidação/redução dos fármacos analisados após a sua religação no MIP produzido. Relativamente ao desenvolvimento do sensor MIP para a deteção eletroquímica da AMT, o mecanismo de deteção baseou-se na reação do sistema tris(2,2'-bipiridina)ruténio(II) ($\text{Ru}(\text{bpy})_3^{2+}$)/AMT.

Os sensores desenvolvidos mostraram uma boa *performance* analítica e permitiram obter uma deteção sensível com excelente seletividade para reconhecer o analito alvo quando comparado com estruturas análogas próximas, que podem ser possíveis interferentes. Adicionalmente, eles foram aplicados com sucesso em amostras de água de rio, água da torneira e água de nascente, demonstrando o seu potencial para serem uma estratégia útil e sustentável para a monitorização e quantificação de fármacos.

Palavras-chave: sensores eletroquímicos, polímeros molecularmente impressos, *design* racional, simulação computacional, modelação molecular, química verde, análise ambiental, água.

List of Publications

Publications in International Peer-reviewed journals

(included in chapters 2 and 3 of the present thesis)

1. **Patrícia Rebelo**, João G. Pacheco, M. Natália D.S. Cordeiro, André Melo, Cristina Delerue-Matos, Azithromycin electrochemical detection using a molecularly imprinted polymer prepared on a disposable screen-printed electrode, *Analytical Methods* 12 (2020) 1486-1494. doi.org/10.1039/c9ay02566a.
2. **Patrícia Rebelo**, Estefanía Costa-Rama, Isabel Seguro, João G. Pacheco, Henri P.A. Nouws, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Molecularly imprinted polymer-based electrochemical sensors for environmental analysis, *Biosensors and Bioelectronics* 172 (2021) 112719. doi.org/10.1016/j.bios.2020.112719.
3. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, André Melo, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Rational development of molecular imprinted carbon paste electrode for furazolidone detection: theoretical and experimental approach, *Sensors and Actuators B: Chemical* 329 (2021) 129112. doi.org/10.1016/j.snb.2020.129112.
4. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Development of a molecular imprinted electrochemiluminescence sensor for amitriptyline detection: from MD simulations to experimental implementation, *Electrochimica Acta* 397 (2021) 139273. doi.org/10.1016/j.electacta.2021.139273.
5. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, André Melo, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, A simple electrochemical detection of atorvastatin based on disposable screen-printed carbon electrodes modified by molecularly imprinted polymer: Experiment and simulation, *Analytica Chimica Acta* 1194 (2022) 339410. doi.org/10.1016/j.aca.2021.339410.
6. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, Isabel Seguro, Maria Natália D.S. Cordeiro, Cristina Delerue-Matos, Computational modelling and sustainable synthesis of a highly selective electrochemical MIP-based sensor for citalopram detection, *Molecules* 27 (2022) 3315. doi.org/10.3390/molecules27103315.

List of presentations in Scientific Meetings

Communications in National and International Scientific Meetings

Oral presentations

1. **Patrícia Rebelo**, João G. Pacheco, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Computer-aided design of molecular imprinted electrochemical sensors for analysis of pharmaceuticals in environmental waters, *Reunião Tripartida FCT-NOVA/ISEP/UA*, Faculdade de Ciências e Tecnologia da Universidade NOVA de Lisboa, Portugal, 23 July 2018.
2. **Patrícia Rebelo**, Isabel Seguro, João Grosso Pacheco, Cristina Delerue-Matos, Sensores eletroquímicos: uma ferramenta poderosa no controle ambiental, *V Workshop de la Red E3TECH - I Workshop Iberoamericano a Distancia E3TECH - "Aplicaciones Medioambientales y Energéticas de la Tecnología Electroquímica"*, Online, 28-31 October 2020.
3. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, André Melo, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Computer-aided design of molecular imprinted electrochemical sensors for analysis of pharmaceuticals in waters, *LAQV Webinars*, Online, 8 September 2021.

Poster presentations

1. **Patrícia Rebelo**, João G. Pacheco, M. Natália D.S. Cordeiro, André Melo, Cristina Delerue-Matos, Azithromycin electrochemical detection using a molecularly imprinted polymer on a disposable sensor, *Water Reuse for a Sustainable Future*, Online, 29-30 June, 2020.
2. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, André Melo, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Molecularly imprinted polymer-based electrochemical sensors for the detection of antibiotics in the environment, *10º Encontro da Divisão de Química Analítica da Sociedade Portuguesa de Química*, Online, 26-28 October 2020.

3. **Patrícia Rebelo**, João G. Pacheco, Iuliia V. Voroshylova, M. Natália D.S. Cordeiro, Cristina Delerue-Matos, Computational modelling and experimental synthesis of a highly sensitive electrochemical MIP-based sensor for citalopram detection in environmental waters, *Biosensors 2021 - The 31st Anniversary World Congress on Biosensors*, Online, 26-29 July 2021.

4. **Patrícia Rebelo**, João G. Pacheco, M. Natália D.S. Cordeiro, André Melo, Cristina Delerue-Matos, Rational development of molecular imprinted carbon paste electrode for furazolidone detection: theoretical and experimental approach, *XXIV Meeting of the Portuguese Electrochemical Society*, Instituto Politécnico de Tomar, Portugal, 20-22 2021.

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List of Abbreviations

2VP	2-vinylpyridine
4VP	4-vinylpyridine
AA	acrylic acid
ABA	4-aminobenzoic acid
AC	activated carbon
ACN	acetonitrile
Ag-N@ZnO	Ag and N co-doped zinc oxide
AHBA	3-amino-4-hydroxybenzoic acid
AIBN	2,2'-azobisisobutyronitrile
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
AMT	amitriptyline
APTES	3-aminopropyltriethoxysilane
ATV	atorvastatin
AuNPs	gold nanoparticles
AZY	azithromycin
BDDE	boron doped-diamond electrode
BPA	bisphenol A
CAP	chloramphenicol
CBZ	carbamazepine
CFX	cefaalexin
CHL	chloroform
CIP	ciprofloxacin
CLOM	clomipramine HCl
CLY	clarithromycin
CN	coordination number
CNT	carbon nanotube
COM	centre-of-mass
CPE	carbon paste electrode
CPF	chlorpyrifos
CTL	citalopram
CTL-NO	citalopram N-oxide
CTL-PA	citalopram propionic acid
CV	cyclic voltammetry

CYP	cypermethrin
d	density
D	self-diffusion coefficient
DA	dopamine
D-CTL	demethylcitalopram
DD-CTL	didemethylcitalopram
DESI	desipramine HCl
DFT	density functional theory
DMF	N, N-dimethylformamide anhydrous
DOX	doxepin HCl
DPV	differential pulse voltammetry
DPASV	Differential pulse anodic stripping voltammetry
E2	17- β -estradiol
EC	emerging contaminant
ECL	electrochemiluminescence
EGDMA	ethylene glycol methacrylate
EIS	electrochemical impedance spectroscopy
ERY	erythromycin
Fe-NCNF	nitrogen-doped carbon nanosheet frameworks decorated with Fe
Fe-PC	Fe-doped porous carbon
FF	force field
FLV	fluvastatin sodium hydrate
FLX	fluoxetine
FTD	furaltadone
FZD	furazolidone
GAFF	general amber force field
GCE	glassy carbon electrode
GO	graphene oxide
GQDs	graphene quantum dot
H-bond	hydrogen bond
HPLC	high performance liquid chromatography
IA	itaconic acid
IF	imprinting factor
IL	ionic liquid
IMI	imipramine HCl
<i>i</i>p	peak current

LC-MS/MS	liquid chromatography in tandem with mass spectrometry
LFX	lomefloxacin
LOD	limit of detection
LOQ	limit of quantification
LOV	lovastatin
LSV	linear sweep voltammetry
LSSV	linear stripping sweep voltammetry
MAA	methacrylic acid
MB	mebendazole
MD	molecular dynamics
MeOH	methanol
MIP	molecular imprinted polymer
MM	molecular mechanics
MNPs	magnetic nanoparticles
MOFs	metal organic frameworks
MWCNT	multi-walled carbon nanotube
NFZ	nitrofurazone
N-GS	nitrogen-doped graphene sheets
NIP	non-imprinted polymer
NORTRI	nortriptyline HCl
NTF	nitrofurantoin
OMNiMIP	one monomer ion imprinted polymer
PATP	o-phenylenediamine
PEDOT	poly(3,4-ethylenedioxythiophene)
PGE	pencil graphite electrode
Ph	phenol
POP	persistent organic pollutant
PRV	pravastatin sodium salt hydrate
PTV	pitavastatin
PVC-COOH	carboxylic polyvinyl chloride
Py	pyrrole
QDs	quantum dot
QM	quantum mechanics
RC	resorcinol
R_{CT}	charge transfer resistance

RDF	radial distribution function
RSD	relative standard deviation
RSV	rosuvastatin calcium
Ru(bpy)₃²⁺	tris(2,2'-bipyridyl)ruthenium(II)
SEM	scanning electron microscopy
SiO₂ NPs	silica nanoparticles
SMV	simvastatin
SMX	sulfamethoxazole
SN	sulfanilamide
SPAuE	screen printed gold electrode
SPCE	screen printed carbon electrode
STRE	streptomycin sulphate
SWAdASV	square-wave adsorptive anodic stripping voltammetry
SWCNT	single-walled carbon nanotube
SWV	square wave voltammetry
TBBPA	tetrabromobisphenol A
TCL	triclosan
TETRA	tetracycline
TRIMI	trimipramine HCl
VMD	visual molecular dynamics
WWTP	wastewater treatment plant
α	selectivity factor
ΔE	binding energy difference
ΔH_{vap}	enthalpy of vaporization

Relevance and Motivation

Pharmaceutical pollution has already become one of the most serious issues on a global scale [1]. Due to their exponential use and incorrect disposal, pharmaceuticals have been discharged from many different routes into the environment [2]. Their widespread presence in aquatic environment is a reality and, WWTPs are considered as the major contributor [3]. Pharmaceuticals are not completely removed in conventional WWTPs [4]. As a result, trace levels of several pharmaceuticals have been found in wastewaters, surface waters, groundwater and, to a lesser extent, drinking waters [1,5]. More importantly, studies have demonstrated that, even at low concentrations, they can directly or via long-term exposure induce adverse effects in many non-target organisms [6]. Among these effects, bacterial resistance has attracted increasing attention, because of the misuse and overuse of antibiotics [7]. Therefore, the development of specific analytical tools to understand the concentrations that occur in aquatic environment is of paramount importance.

High performance liquid chromatography (HPLC) coupled with UV or mass spectrometry detection is by far the most widely used technique for the determination of pharmaceuticals in several matrices [1]. Besides its excellent performance, HPLC technique is in most cases too expensive and processing chromatograms is laborious and time consuming. Alternative methods allowing rapid response, simplicity, low cost, miniaturisation and direct analysis are essential when the final goal is the application in real sample monitoring. At the same time, it is also important to consider new approaches that comply with green analytical principles.

Electrochemical sensors are devices that provide information about the composition of a system in real time, which can fulfill all these requirements [8]. Their combination with MIPs has led to wide applications in analytical chemistry [9,10]. However, there is still a lack of electrochemical MIPs sensors to detect pharmaceuticals in environmental waters.

Thesis objectives

The main goal of this thesis was the development of sensitive and highly selective electrochemical MIPs-based sensors for the detection of pharmaceuticals in environmental waters. For this purpose, a combination of theoretical and computational methods was applied to achieve a rational MIP design, as well as to better understand key interactions occurring in the polymerisation mixture. In this context, different intermediate objectives were aimed to be reached:

- Get a fast, easy to use, low cost and environmentally friendly electrochemical MIPs sensors;
- Apply computational studies for the preparation of MIPs and contribute to novel approaches to achieve better imprinting factors;
- Develop and optimise different polymerisation methods aiming to minimise the amount of organic solvents and waste generated;
- Develop of detection strategies coupled with nanomaterials to amplify electrochemical responses and guarantee low limits of detection;
- Validate and evaluate electrochemical MIP sensors for the detection and quantification of pharmaceuticals in water samples.

Thesis structure and organisation

This thesis concerns the work developed during the last four years under the scope and objectives of the approved PhD project. It is organized into four major chapters, and the contents of each chapter is summarised as follows:

CHAPTER 1 – General Introduction

This chapter provides a general contextualisation of the research focus. Firstly, the vulnerability of environmental waters to emerging contaminants (ECs) with a specific attention to contamination caused by the release of pharmaceuticals is presented. Following, the role of WWTPs is discussed by pointing out different stages in which pharmaceuticals may be resistant to degradation and enter into aquatic systems. Current European water legislation and conventional detection of pharmaceuticals are sections driving to the importance of electrochemical sensors combined with MIPs for monitoring these compounds. Finally, this section also includes a brief review of computational studies accompanied by experimental works that have been reported in the design of electrochemical MIPs sensors for the detection of pharmaceuticals.

CHAPTER 2 – Electrochemical sensing of environmental contaminants via molecularly imprinted polymers

This chapter consists of an extensive review paper addressing the construction of electrochemical MIP sensors with focus on the polymerisation technique, functional monomers, sensing platforms and materials employed to achieve the best sensitivity. A summary of the main environmental contaminants (pharmaceuticals, pesticides, heavy metals and other) is provided, including an overview of the MIP-based electrochemical sensors that have been reported for the detection of these contaminants in water samples over the past decade. Here, the performance of the different sensors is compared, including the limit of detection (LOD), the linear concentration range and the type of water samples analysed.

CHAPTER 3 – Rational development of electrochemical MIPs sensors for pharmaceuticals detection

This chapter is devoted to all experimental work developed, encompassing five research papers about the construction and design of distinct electrochemical MIP-based sensors for the detection of pharmaceuticals playing a pivotal role in medical

care and frequently detected in environmental waters. All these works employ computational studies, namely QM calculations and/or MD simulations for a MIP design protocol.

CHAPTER 4 – Final conclusions and future perspectives

This last section contains the main conclusions of the work and presents challenges and future prospects for the use of electrochemical MIPs sensors for environmental analysis.

The papers included in chapter 2 and 3 were published during the period of the project and, the original content and structures organisation were maintained according to the journal in which they were published and with request permission.

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Chapter

1

GENERAL INTRODUCTION

1.1 Water pollution – Emerging contaminants

1.2 Pharmaceutical water pollution

1.2.1 The role of WWTPs

1.2.2 European legislation

1.2.3 Conventional detection of pharmaceuticals

1.3 Electrochemical MIP-based sensors

1.3.1 Computational procedure for the design of MIPs

References

1.1 Water pollution - Emerging contaminants

Environmental pollution is one of the most serious consequences caused by human action in our planet. Population growth, overconsumption and industrialisation are polluting world's resources, that have been under threat, at an alarming proportion. As a direct consequence of these anthropogenic activities, the amount of waste generated has been increasing every year, and the management of its disposal is a global challenge.

Water, a pivotal and irreplaceable resource to sustain human life, has become an environmental compartment intensively used and altered by pollutants of various categories (pathogens, organic and inorganic chemicals), which are explicitly or implicitly discharged and accumulated into different water bodies. The deterioration of water quality is a reality with multiple human health and ecosystems risks [1]. And, with the increasing demand for safe and clean water, water pollution is now among the major environmental and health concerns, which has attracted widespread interest from governments and the scientific community.

Owing to their persistence in environmental degradation and biomagnification through trophic levels, persistent organic pollutants (POPs) are the classical compounds that have long been considered in environmental water monitoring studies. Examples of “traditional” or well-known POPs include toxic heavy metals, pesticides and polyaromatic hydrocarbons [2]. However, studies on wastewater characteristics, in consequence of advancements in analytical instrumentation, have reported substances that were not detected before [3]. Some of these are considered emerging contaminants (ECs) typically defined as “Pollutants currently not included in routine monitoring programmes at Union level but which could pose a significant risk requiring regulation, depending upon their potential ecotoxicological and toxicological effects and on their levels in the aquatic environment” (Directive 2013/39/EU) [4].

ECs are included in a long list of products used daily by humans that can be categorised into three major classes according to their physicochemical properties: organic compounds, inorganic compounds and particulate contaminants (Figure 1) [5].

Most of non-regulated substances have entered the environment for years, even decades. However, their extensive use and ubiquity in rivers, lakes and water supply reservoirs have prompted the classification of ECs as hazardous water pollutants [6,7]. In this direction, ECs have been the subject of several investigations, establishing their potential for negative ecotoxicological effects at concentrations as low as few ng L⁻¹. In particular, a number of efforts has been made to highlight the need for regulation and

monitorisation of ECs with a focus on water systems aiming to prevent and minimise their impacts on “One Health” trilogy (environment, human and animal health) [8].

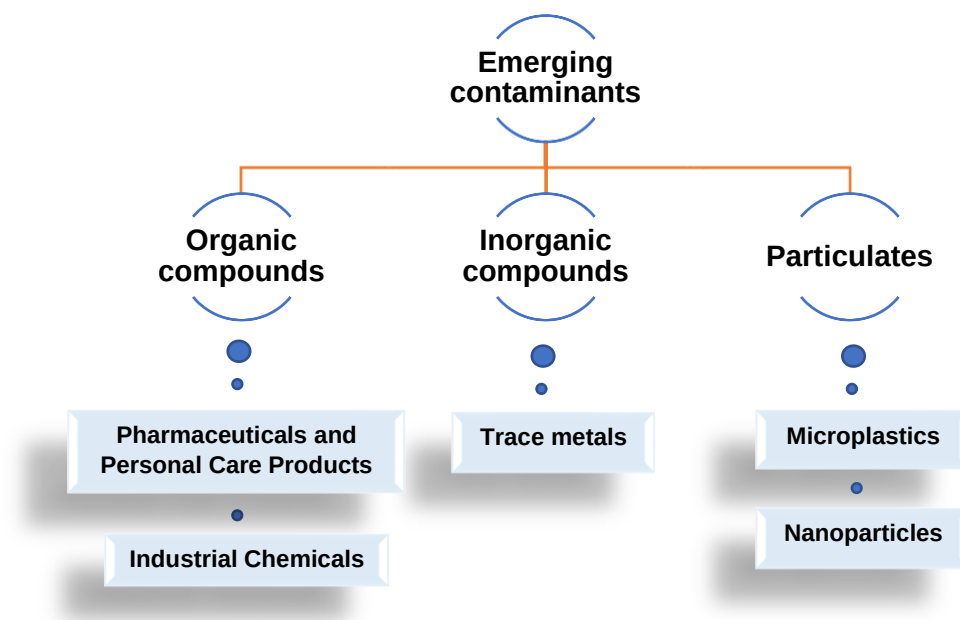


Figure 1. Simplified characterisation of the ECs classification scheme. Adapted from [5] with permission from Elsevier.

1.2 Pharmaceutical water pollution

Among ECs, pharmaceuticals represent a category of wide interest [9]. They include a set of compounds of environmental concern like antibiotics, lipid regulators, antidepressants, analgesics and nonsteroidal anti-inflammatory drugs commonly used as a part of human and veterinary medication or to promote growth in livestock. Pharmaceuticals play an undisputed role in supporting people’s health and lives, and consequently, their consumption is growing driven by the need to prevent ageing-related and chronic diseases of the modern world [10]. Global antibiotic consumption increased by 65% between 2000 and 2015 [11], prescription of lipid regulators, including statins which are very important in prevention of cardiovascular diseases, had nearly quadrupled, and the consumption of antidepressants doubled from 2000 to 2017 in Organization for Economic Cooperation and Development countries [10,12,13]. Considering that these compounds have been intensively prescribed or sold improperly and used, it is not surprising to observe their occurrence in environmental water matrices from different continents of the planet (Figure 2).

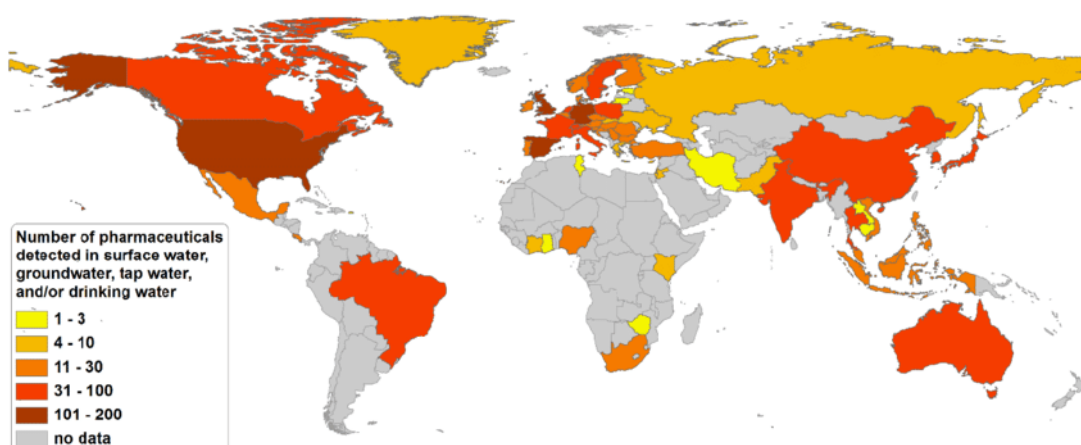


Figure 2. Number of different pharmaceuticals detected in surface water, groundwater and/or tap water around the world. Reproduced from [14] with permission from John Wiley and Sons.

The percolation of many active pharmaceutical ingredients has been frequently described in surface water, which includes lakes, rivers, streams, estuaries and seawater, groundwater, drinking water, as well as in Wastewater Treatment plants (WWTPs) effluents and influents [15]. Despite many pharmaceuticals not behaving as POPs, they are being released at rates exceeding environmental degradation, which makes them pseudo persistent in waters [16]. Additionally, it is important to emphasise that these compounds are designed to interact with specific processes of target molecules producing a therapeutic response at low doses. In this regard, it is consensual that even the trace levels of pharmaceuticals found and reported in water compartments (ranging from ng L^{-1} to $\mu\text{g L}^{-1}$) can produce undesirable effects on non-target organisms enhanced by chronic exposure and mixture of several compounds [15].

Environmental exposure to pharmaceuticals can occur through various emissions from urban wastewater, pharmaceutical industry, agricultural run-off and illegal discharges [14]. Nevertheless, WWTPs are currently considered as the primary transport pathway of ECs into the environment, mainly in surface water [17,18]. Because of their unique physicochemical properties (stability, volatility, high polarity and lipophilicity and adsorption), diversity and low concentration, the removal of pharmaceuticals at WWTPs is a complex process [19]. Although their impact on environmental water have decreased over time, WWTPs were not designed to remove pharmaceuticals and, as new pollutants are found, new challenges will always arise [20].

1.2.1 The role of WWTPs

The conventional wastewater treatment typically employed in WWTPs can be divided into three encompassing steps aptly known as primary, secondary and an optional or advanced tertiary treatment [21]. Primary treatment is a process intended to remove suspended and colloidal solids by adsorption. In this step the removal efficiency of pharmaceuticals is highly affected by their hydrophilic nature and the chances of getting adsorbed are very limited [22]. There is a low degradation of pharmaceuticals in primary treatment, where most of them remain unchanged in the aqueous phase of the treated effluent [19].

The secondary treatment stage aims at removing solid organics that escape the primary step and further removal of suspended solids mainly through biological degradation mechanisms (aerobic or anaerobic) [23]. Activated sludge processes are the most secondary applied procedure in conventional WWTPs due to both their performance and low operation costs. Nevertheless, these systems present a wide range of pharmaceutical removal efficiencies, which are largely dependent on operating conditions, such as hydraulic retention time, pH and initial concentration of contaminants [24,25]. Obviously, not all WWTPs have the same wastewater composition entering the plants and, pharmaceuticals arrive in unpredictable amounts and interval time [26], which makes the removal process an easily underestimated protocol.

To define the most suitable biological treatment it is important to consider wastewater characteristics. While biological processes can act as key contaminant removal tools with several studies showing their ability to provide significant reductions in the pharmaceutical concentrations [23,27], some compounds exhibit an extremely persistent and recalcitrant character [28,29]. As an example, diclofenac is hardly biodegraded during secondary treatment and, consequently, it is a dominant pharmaceutical in WWTPs effluents [19,30]. It is also known that pharmaceuticals, mostly antibiotics, can induce toxicity on microbial community in activated sludge, thereby inhibiting the process of degradation [22]. Negative removal efficiency values, where concentrations are lower in the WWTPs influent compared with the treated effluent, has also been reported for pharmaceuticals like carbamazepine [24,31]. Several reasons have been attributed to this phenomenon, and one of the most cited describes the presence of unmeasured conjugated compounds that through enzymatic cleavage are retransformed into their original parent compound during biological processes [21,31].

Taking the aforementioned into account, it seems evident that improved treatments are needed to guarantee better inactivation, degradation and removal of

persistent compounds. In this line, tertiary treatment or advanced treatment processes have been used as a polishing step past the secondary treatment. Many efforts have been reported in the literature, and among others, activated carbon and ozonation have shown promising results for upgrading discharges of WWTPs when they work under optimum conditions [32,33]. Although these processes have higher potential for pharmaceutical removal in comparison with conventional treatments, they are not widely used due to their cost linked to high energy demand [34]. On the other hand, the generation of toxic oxidation by-products, particularly during ozonation process, has also been discussed, since they may sometimes be more toxic and persistent than their parent compounds [21,35]. Therefore, it can be concluded that regions with a poor or inadequate wastewater management system are more vulnerable to emerging contaminants, and consequently, pharmaceuticals.

The lack of proper guidelines and regulations, limiting the levels of pharmaceuticals in environmental waters has masked the severity of pharmaceutical contamination over the years. In order to reverse this situation, many countries across the globe have worked on the implementation of policies, guidelines or measures to address the issue of pharmaceuticals in the aquatic environment [19]. However, pharmaceuticals remain on a Watch List up to the present time.

1.2.2 European legislation

In 2000, the European Union established the Water Framework Directive, which contains the surface water Watch List of substances/groups of substances that shall be regularly monitored by EU Members States for the purpose of defining the risk they pose to or via the aquatic environment, and support their prioritisation [19,36].

The Directive 2000/60/EC was the first step towards sustainable management of European freshwater resources [37,38]. According to this directive, all water bodies across the EU should have achieved good chemical status and ecological potential by 2015, and 2027 at the latest [39]. These efforts require specific measures in order to identify and quantify several pollutants, progressively reducing discharges, emissions and losses of priority substances or completely phase out priority hazardous pollutants from water ecosystems [40]. Accordingly, a set of 33 priority substances and their respective environmental quality standard were introduced by the ratified Directive 2008/105/EC. This Directive was posteriorly revised by the Directive 2013/103/EU, recommending the monitorisation and treatment options for 45 priority substances.

Furthermore, it was proposed at the first instance the establishment of a dynamic and limited watch list, comprising two pharmaceuticals, namely, diclofenac and the synthetic hormone 17-alpha-ethinylestradiol [37,41].

The definitive first EU Watch list was published in 2015 with a total of 17 ECs (Decision 2015/495/EU), which was updated by Decisions 2018/840/EU and 2020/1161/EU [42]. Besides the abovementioned pharmaceuticals, the referred Watch List also contemplated the macrolide antibiotics azithromycin, erythromycin and clarithromycin for a continuous monitoring for up to four years [37]. Since sufficient available monitoring data was gathered to carry out an appropriate diclofenac risk assessment, this pharmaceutical was removed from the Watch list in 2018 [43]. At the same time, amoxicillin and ciprofloxacin were added, highlighting the concern raised about antimicrobial resistance [44]. Recently, the Commission under Decision 2020/1161/EU in accordance with the new EU Strategic Approach to Pharmaceuticals in the Environment and the European Action Health Plan decided to include two antibiotics in the third Watch List, sulfamethoxazole and trimethoprim, the antidepressant venlafaxine together with its metabolite O-desmethylvenlafaxine and three azole fungicides used as pharmaceuticals, clotrimazole, fluconazole and miconazole, while synthetic hormone 17-alpha-ethinylestradiol and macrolide antibiotics were excluded [19,45].

Undoubtedly, this initiative presents important steps taken to manage and limit the release of pharmaceuticals into the environment, as well as highlight the need for research on suitable approaches to expand drug monitoring [41,45]. However, the number of studied pharmaceuticals is still limited and repetitive. Literature has shown that more than the most frequent pharmaceuticals studied should be considered. There is a set of compounds that due to its role in changing world might be potential candidates for future regulations of a more sustainable water policy. So, pharmaceuticals monitoring should continue to be a priority.

1.2.3 Conventional detection of pharmaceuticals

The knowledge of the pharmaceuticals and their concentrations on the aquatic environment is one of the most crucial tasks towards implementing mitigation strategies. In fact, the advancement in our acknowledgement of the widespread presence of pharmaceuticals is clearly correlated with the progress in the field of analytical instrumentation leading to greatly improved sensitivity, selectivity, robustness and reproducibility/repeatability analysis.

Liquid chromatography in tandem with mass spectrometry (LC-MS/MS) is the most applied technique for the detection and identification of pharmaceuticals in environmental matrices [15,46–48]. On the one hand, LC-MS/MS is able to provide high sensitivity and selectivity data. On the other hand, this technique commonly requires extensive steps of sample preparation to achieve the same goal and to not miss the target. LC-MS/MS is also limited by all the costs and long analysis time associated to the analytical procedure [49]. As a result, the scientific community has devoted particular attention to the development of low-cost sample handling approaches to pharmaceuticals detection, allowing not only for simple and real-time analysis, but also for miniaturisation of analytical methodologies in order to reduce the amounts of reagents or solvents employed and, consequently, the amount of waste generated [50]. In view of the limitations associated with sample collection and subsequent laboratorial analysis, the availability of assays and devices enabling portability is also highly desirable for continuous environmental monitoring [51].

1.3 Electrochemical MIP-based sensors

Devices based on electrochemical detection are well established techniques, and chemical sensors, namely electrochemical sensors have emerged as valuable instruments to address the challenges of analytical chemistry. Extensive and recent revisions have highlighted their potential to detect low concentrations of different analytes for environmental application, including pharmaceuticals [52,53]. Comparable or even better than the conventional analytical systems, electrochemical sensors can be easily automated and miniaturised, resulting in shorter analysis time and low volume samples.

Many methods can be taken into consideration to design electrochemical sensors combining a biological element, like enzymes or antibodies, with a physical transducer. Despite its relevant performance, the usage of biological elements is known for its low stability and high cost, which limits their application. In order to spawn new fields of use, a new generation of electrochemical sensors, based on a concept of artificial receptors has seen an increase in high-quality publications over the last years [54]. Molecularly imprinted polymers (MIPs) represent one of the most promising alternatives. Operating by a “lock and key” mechanism to selectively bind the desired analyte, MIPs based on electrochemical sensors have clearly demonstrated their abilities to achieve the selectivity and sensitivity of the biological receptors, benefiting from a simple synthesis,

low cost and versatility in terms of working conditions (pH, temperature, organic solvents) [55]. Consequent to these remarkable properties, MIPs integrated into electrochemical sensing devices benefit from significant advantages in real-sample monitoring.

Regarding the trends in recent years, works involving electrochemical MIPs and pharmaceuticals detection have attracted attention from the scientific community [53]. However, their application in water samples remains an open challenge.

MIPs synthesis involves a molecular imprinting process based on the interaction between a suitable functional monomer and the target molecule through covalent or non-covalent bonds. This procedure that introduces a “molecular memory” into a 3D polymer network is in principle simple. However, finding the best synthesis parameters, including the most appropriate composition for optimal performance, can be a laborious and time- and resource-consuming task [56]. As a result, much attention goes to the use of computational techniques in the study and development of MIPs.

1.3.1 Computational procedure for the design of MIPs

Computational approaches have been employed not only to understand the underlying molecular level mechanisms of molecular imprinting recognition, but also to assist in the rational MIP design allied with the concept of green analytical chemistry. Many studies correlated with experimental results have shown their great potential in functional monomer screening, selection of cross-linker and solvent, optimisation of functional monomer:template ratio and selectivity analysis [57,58].

Quantum mechanics (QM), molecular mechanics (MM) and molecular dynamics (MD) simulations are the most frequently applied methods for MIPs design [58,59]. QM calculation approaches, namely semi-empirical, *ab initio* and density functional theory (DFT), have been mainly used by researchers for the precise analysis of functional monomer-template interactions to predict molecular energies with electronic structure-based techniques [59]. At the theoretical level, there is special focus on the search of an ideal monomer able to establish strong and stable affinities with the intended template for a better imprinting efficiency [60]. Among them, DFT methods have become more widespread in MIPs design, thanks to their simplicity, high accuracy and cost-effective nature [57,58]. Based on the lowest energy as an indicative of the strongest interaction, the binding energies differences (ΔE) between the template and different functional monomers are generally calculated by the equation (1):

$$\Delta E = E(\text{complex}) - E(\text{template}) - E(\text{monomer}), \quad (1)$$

where $E(\text{complex})$ is the association energy of template-monomer complex, $E(\text{template})$ and $E(\text{monomer})$ denote the energy of template and functional monomer involved in the complex, respectively.

Although QM-based methods provide good predictive capacity, the increase in the complexity of the pre-polymerization system is proportionally accompanied by an increase in the computational complexity, which reduces their validity in representing all-component pre-polymerization system. [56]. Hence, MM and MD are typically preferred methods, since they allow modelling multi-component systems with thousands of atoms at reasonable computation expense and time [57]. Using energy functions to inquire about molecules properties, MM and MD are having an important role on the development of rational MIPs [56,57]. While MM treat molecules as a collection of atoms, ignoring their movements, MD simulate the time evolution of a set of interacting atoms in space with a specific temperature and pressure, and gives the possibility of analyzing the motion of specific molecules [59]. MD provide a more realistic simulation, where the components and concentrations used experimentally for polymer synthesis can be reproduced with solvent molecules explicitly included. These are a powerful tool to study the structure, dynamics and energetic of different molecular systems that can be subsequently extracted and correlated with MIP recognition performance. However, considering that each computational technique offers unique information, the combined use of QM calculations and MD simulations seems advantageous to explore a multitude of variable parameters taking place in pre-polymerisation mixtures [61].

Specifically, in relation to design electrochemical MIP sensors for pharmaceuticals detection, the analysis of literature review over the last decade suggests continuous efforts on application of computational approaches for screening functional monomers and to study interactions involved in the pre-polymerization mixture (Table 1). Table 1 shows a special focus on QM calculation by DFT methodology, present in literature. On the other hand, few MD studies have been reported. Despite extensive investigations on MD simulations to design MIPs, this approach still remains unexplored to construct MIP-based electrochemical sensors for pharmaceuticals assessment.

Table 1. Computational studies for the rational design of electrochemical MIP sensors to detect pharmaceuticals.

Computational simulation	Details	Template	MIPs design/application
Quantum Mechanics (QM)	DFT	Methimazole [62], celecoxib [63], hydrochlorothiazide [64], valaciclovir [65], famciclovir [66], sofosbuvir [67], minoxidil [68], ethionamide [69], metoprolol [70], lamotrigine [71], sulfadimidine [72], triamterene [73], 6-thioguanine [74], enzalutamide [75], lansoprazole [76], mosapride citrate [77], pantoprazole [78], metformin hydrochloride [79], acetaminophen [80], chlortetracycline [81], primaquine [82], erythromycin [83], cilostazol [84], azithromycin [85], furazolidone [86], atorvastatin [87]	Screening of functional monomers, template:monomer ratio and evaluation of pre-polymerisation mixture properties
	<i>Ab initio</i>	2-Aminobenzimidazole [88]	Screening of functional monomers
Molecular Mechanics (MM)	CHARMM, Tripos	Cilostazol [84], sitagliptin [89]	Screening of functional monomers
Molecular Dynamics (MD)	OPLS, CHARMM, GAFF	6-Thioguanine [74], cilostazol [84], furazolidone [86], atorvastatin [87], amitriptyline [90], citalopram [91]	Screening of functional monomers, solvent, template:monomer ratio and evaluation of pre-polymerisation mixture properties

DFT: Density Functional Theory; CHARMM: Chemistry at Harvard Macromolecular Mechanics; OPLS - Optimised Potential for Liquid Simulations; GAFF: General Amber Force Field

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Chapter

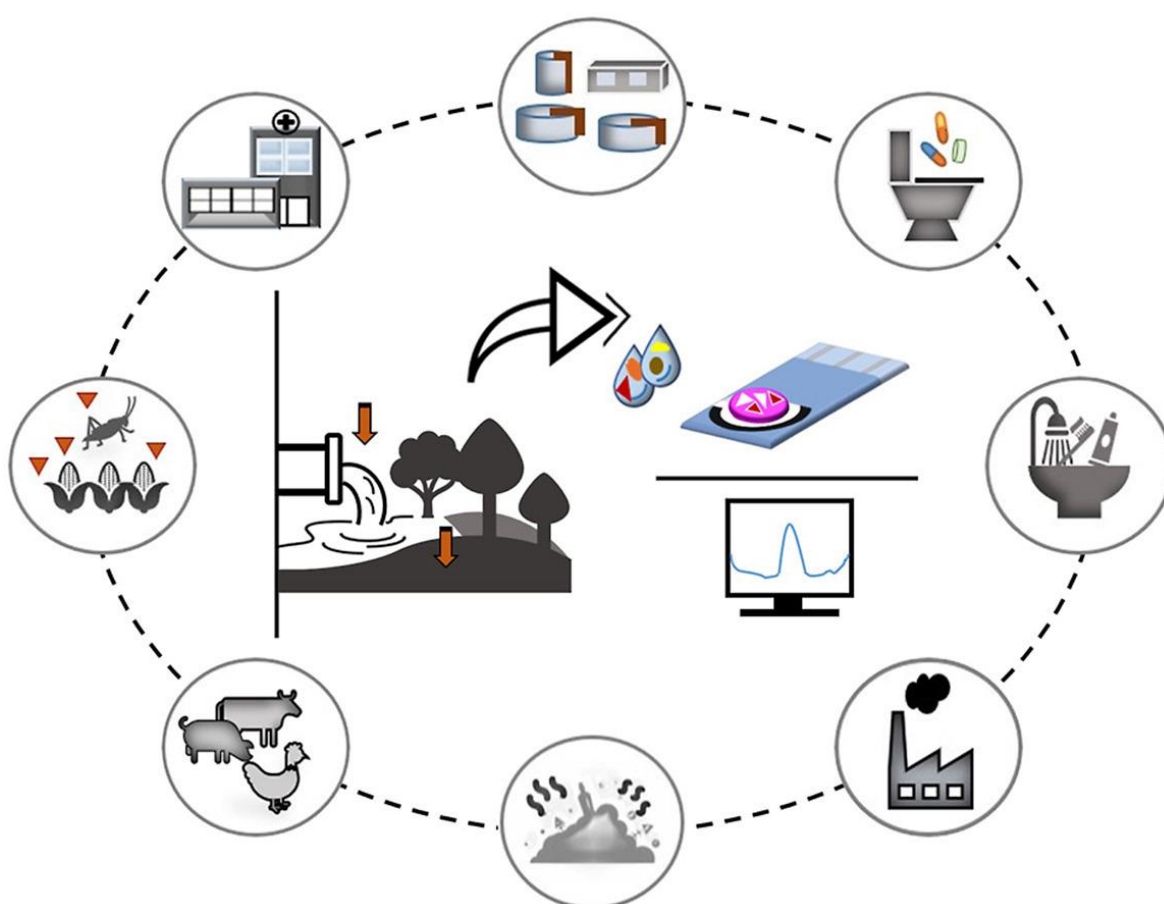
2

ELECTROCHEMICAL SENSING OF ENVIRONMENTAL CONTAMINANTS VIA MOLECULARLY IMPRINTED POLYMERS

2.1 Molecularly imprinted polymer-based electrochemical sensors for environmental analysis

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Biosensors and Bioelectronics 172 (2021) 112719



Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review on electrochemical MIP sensors reported for the detection of pharmaceuticals, pesticides, heavy metals and other contaminants in water samples in the past decade and writing of the original draft.

Highlights

- Contaminant analysis faces great challenges because of their variety and levels.
- Advantages of MIPs as recognition elements: low-cost, robustness, reusability.
- Main inconvenient of MIP-based sensors for environmental analysis: sensitivity.
- Discussion of MIP-based electrochemical sensors for environmental analysis.
- Discussion of advances and trends in MIP-based electrochemical sensors.



Molecularly imprinted polymer-based electrochemical sensors for environmental analysis

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ARTICLE INFO

Keywords:

Molecularly imprinted polymer
Electrochemical sensors
Emerging contaminants
Pharmaceuticals
Pesticides
Heavy metals

ABSTRACT

The ever-increasing presence of contaminants in environmental waters is an alarming issue, not only because of their harmful effects in the environment but also because of their risk to human health. Pharmaceuticals and pesticides, among other compounds of daily use, such as personal care products or plasticisers, are being released into water bodies. This release mainly occurs through wastewater since the treatments applied in many wastewater treatment plants are not able to completely remove these substances. Therefore, the analysis of these contaminants is essential but this is difficult due to the great variety of contaminating substances. Facing this analytical challenge, electrochemical sensing based on molecularly imprinted polymers (MIPs) has become an interesting field for environmental monitoring. Benefiting from their superior chemical and physical stability, low-cost production, high selectivity and rapid response, MIPs combined with miniaturized electrochemical transducers offer the possibility to detect target analytes in-situ. In most reports, the construction of these sensors include nanomaterials to improve their analytical characteristics, especially their sensitivity. Moreover, these sensors have been successfully applied in real water samples without the need of laborious pre-treatment steps. This review provides a general overview of electrochemical MIP-based sensors that have been reported for the detection of pharmaceuticals, pesticides, heavy metals and other contaminants in water samples in the past decade. Special attention is given to the construction of the sensors, including different functional monomers, sensing platforms and materials employed to achieve the best sensitivity. Additionally, several parameters, such as the limit of detection, the linear concentration range and the type of water samples that were analysed are compiled.

1. Introduction

Pollution is defined as the direct or indirect introduction of substances or energy into the environment, by man, that are liable to cause harm to human health, living resources or the biosphere's ecological and physical systems (Holdgate, 1979; Khanmohammadi et al., 2020; Manisalidis et al., 2020). Although pollutants can also enter the environment through natural processes, such as volcanic activity, this definition, contained in EU legislation (Directive, 2008/1/EC), assigns environmental responsibilities to human activities. Besides the evident detrimental effects (such as loss of biological diversity, introduction of invasive species, excessive amounts of hazardous chemicals in the food chain, and global climate change), environmental pollution is a major

cause of illness that leads to great economic costs for health care systems. The terms pollution and contamination are sometimes used as synonyms but they are not really the same. A contaminant is a substance that is not normally present in the environment or that is present in concentrations above the natural background but is not necessarily harmful. On the other hand, a pollutant is a contaminant that results in harmful effects to the environment or living organisms (Chapman, 2007). However, in some cases this distinction is a bit fuzzy because harmful effects may occur but are not observed. Over the past decades, the rise in population, globalisation, and industrialisation have led to the dissemination and the increase of the amount and the variety of hazardous substances in the environment. The harmful effects to living organisms and the ecosystem of many of these substances, which range

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<https://doi.org/10.1016/j.bios.2020.112719>

Received 26 August 2020; Received in revised form 7 October 2020; Accepted 10 October 2020

Available online 20 October 2020

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from heavy metals and pesticides to phenolic compounds, xenobiotics, plasticisers and antibiotics, have been demonstrated (Chakraborty et al., 2020; Dulio et al., 2018; Richardson and Ternes, 2014). However, there is still insufficient knowledge about the toxicity of some of them to environmental, animal and human populations.

Among these substances, the so-called “contaminants of emerging concern” (CECs) or “emerging contaminants” are receiving huge attention because of their increasing presence and extensive distribution in the environment (Dulio et al., 2018; Kroon et al., 2020; Naidu et al., 2016; Petrie et al., 2014). CECs, and their metabolites and/or transformation products, are a heterogeneous group of compounds that are present in a wide variety of products, including industrial chemicals, pharmaceuticals, drugs of abuse, personal care products, biocides, food additives, surfactants and plasticisers (Dulio et al., 2018; Lapworth et al., 2012; Ramírez-Malule et al., 2020). The term CECs does not only cover newly developed substances but also chemicals that have entered the environment for years, whose presence has only recently been detected (either because of the increase of their concentration or because of the advances in analytical methods) and/or studied (Richardson and Ternes, 2014; Zulkifli et al., 2018). Due to insufficient knowledge about the toxicity, impact, and behaviour of CECs, many of them are not yet regulated, so they are not routinely monitored and submitted to an emission control regulation. However, since their potential negative effects are increasingly recognised, relevant regulations are expected over the next years (Bilal et al., 2019). The variety and amount of CECs is so impressive that the list of priority substances created by NORMAN (the network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances funded by the European Commission) includes more than 900 (Dulio et al., 2018; norman-network.).

Although CECs can enter the environment by the same routes as traditional contaminants, such as industrial processes and emissions, wastewater is recognised as a highly important contributor to the entry of CECs in the aquatic environment (Lapworth et al., 2012; Luo et al., 2014; Paiga et al., 2016), especially in countries/regions which do not have suitable regulation regarding wastewater treatment. However, even in regions with adequate regulation, removal of CECs may be incomplete because this largely depends on the physical-chemical characteristics of the contaminants and on the treatments applied in the wastewater treatment plants (WWTPs) (Lapworth et al., 2012; Luo et al., 2014; Patel et al., 2019). Other important sources of the release of CECs into the aquatic environment are landfill sites, since CECs may leach and reach groundwater, sewage sludge, and manure (that may contain veterinary pharmaceuticals) used in agriculture (Lapworth et al., 2012; Sui et al., 2015). Therefore, the determination of CECs levels in the aquatic environment is of great importance, not only to evaluate the water quality and security, but also to improve the knowledge on their pathways and fate.

As mentioned previously, the increasing amount and variety of contaminants has led to more stringent environmental regulations and, consequently, to the increase of the demands on environmental analysis. Vice versa, the latest improvements in environmental analysis results in legislation stringency. The analysis of contaminants in waters involves great challenges since they are a very large group with very different physical/chemical properties that are present at very low concentrations (Geissen et al., 2015; Rasheed et al., 2019). In this context, powerful analytical techniques (e.g. high-performance liquid chromatography and gas chromatography coupled to mass spectrometric detection (HPLC-MS and GC-MS, respectively) (Arismendi et al., 2019; Beldean-Galea et al., 2020; Benedetti et al., 2020; Liang et al., 2020; Merlo et al., 2020; Paiga et al., 2019) are used for the detection of contaminants in waters. However, these techniques are often not as fast and cost-effective as desired since they require complex and expensive instrumentation, laborious sample pre-treatment procedures and long analysis times (Rasheed et al., 2019; Zulkifli et al., 2018). Spatial and temporal variations are also important issues in environmental analysis

due to seasonality, inter- and intra-day variations and occasional events that can hamper a harmonised and representative screening (Luo et al., 2014; Paiga et al., 2019). Therefore, complete information of the presence of contaminants in water bodies and its spatial and temporal distribution requires the collection of several samples at different times. When conventional analytical methodologies, such as solid phase extraction followed by chromatographic analysis (Kuhn et al., 2020), are used, these samples must be sent to a laboratory, turning real-time information impossible. Hence, there is the need to develop technological solutions for easy, fast and on-site contamination monitoring that provide a representative spatial-temporal picture of environmental quality.

The growing demand for inexpensive and easy-to-use analytical devices capable of rapidly providing valuable qualitative and quantitative on-site information increases the interest in electrochemical sensors because of their reduced size, portability, low cost and low reagent and sample consumption. Moreover, the combination of cutting-edge technologies in digital communication networks and innovative sensors allows the construction of smart analytical tools. A robust electrochemical sensor requires the successful integration of a transducer (usually an electrochemical cell comprising a working- a reference- and a counter electrode) with a recognition element (e.g. biological (enzyme, antibody, protein ...) or artificial (e.g. molecularly imprinted polymers (MIPs)). MIPs have attracted wide attention as recognition elements for sensor development due to their high selectivity towards the target analyte and their advantages compared to biological receptors: i) easy and low-cost preparation, ii) physical and chemical robustness when unfavourable conditions are used, such as organic solvents, extreme pH values, high temperatures and/or high pressures, iii) reusability, iv) stability and v) possibility of large scale production (Ansari and Karimi, 2017a; Figueiredo et al., 2016; Gui et al., 2018).

The basis of today's molecular imprinting technology (MIT) started with the studies presented by Wulff and Sarhan (1972) almost 50 years ago (Beluomini et al., 2019). In the first decades the development and application of MIPs were mainly focused on separation and extraction techniques. Nowadays, MIPs are used in a wide variety of applications (Belbruno, 2019; Chen et al., 2016) including sample preparation (e.g. solid phase extraction) (Ansari and Karimi, 2017b), chromatographic separation (Boysen, 2019; Yang et al., 2016), drug delivery (Han et al., 2019; Luliński, 2017; Mokhtari and Ghaedi, 2019) and chemical sensing (Cai et al., 2019; Lopes et al., 2017; Pacheco et al., 2018; Rebelo et al., 2020). Electropolymerisation has facilitated the application of MIPs in electrochemical sensing by employing electroactive monomers that can polymerize by applying electric current. The first studies of selective MIPs towards several species produced in this way were done by ; Boyle et al. (1989), Dong et al. (1988) and Vinokurov (1992). After this, the first combination of electropolymerised MIPs and sensors was described by Hutchins and Bachas (1995) for the analysis of nitrate using a potentiometric sensor. The electrochemical synthesis approach was also successfully used to develop MIP-sensors by two different groups in 1999 (Deore et al., 1999; Malitesta et al., 1999). Malitesta et al. (1999) proposed the electropolymerisation of o-phenylenediamine (o-PD) in the presence of glucose (neutral molecule) in an aqueous environment on a gold-coated quartz crystal to assemble a piezoelectric sensor. Deore et al. (1999) reported the electrochemical preparation of polypyrrole (PPy) imprinted with L-glutamate anion. Along with electropolymerisation, the modification of electrodes with MIPs prepared by traditional methods such as bulk polymerisation was also explored. More recently, the MIP technology has been coupled to disposable and miniaturized screen-printed electrodes. So, in the future it is foreseen that some MIPs could be used in commercial sensing solutions.

Theoretically, MIPs can be prepared for any molecule of interest; indeed, the MIP database (available at <http://nipdatabase.com>) compiles MIPs for more than 10,000 target molecules. Therefore, in the last decade, the development of MIP-based electrochemical sensors has exploded as well as the concern about emerging contaminants (Fig. 1) (Ahmad et al., 2019; Ansari and Karimi, 2017a; Beluomini et al., 2019;

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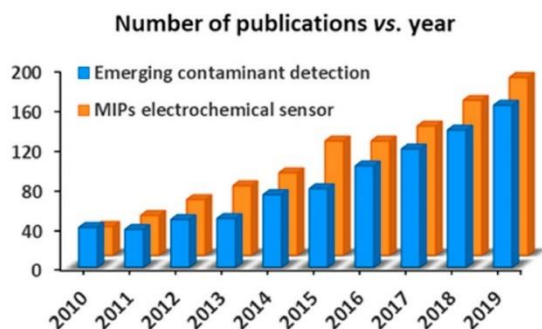


Fig. 1. Evolution of publications regarding MIP-based electrochemical sensors and emerging contaminant detection in the last 10 years.

Gui et al., 2018; Lahcen and Amine, 2019). Combining MIPs and electrochemical sensing strategies has shown great potential to benefit environmental pollution control (Ayankojo et al., 2020).

In this work, a comprehensive review of MIP-based electrochemical sensors for the analysis of contaminants in water is provided. There are some recent reviews about this kind of sensors, however, they are general regarding the applications of the sensors (Beluomini et al., 2019; Gui et al., 2018; Lahcen and Amine, 2019), or they are focused on the preparation and use of imprinted polymers for recognition and extraction, not only in sensing applications but also in solid phase (micro) extraction (Ansari and Karimi, 2017a; Figueiredo et al., 2016). Thus, this review includes a summary of the main environmental contaminants and provides a general overview of the MIP-based electrochemical sensors reported for the analysis of these contaminants in water samples focusing on the polymerisation technique, functional monomers, types of electrodes and electrode modifiers and comparing their performances. Finally, the present challenges and prospects for advancing the use of this kind of sensors for environmental analysis are discussed.

2. MIPs as recognition elements for electrochemical sensors

MIPs are tailor-made synthetic materials with selective binding cavities that act as recognition sites for a specific target molecule, mimicking natural receptors (Chen et al., 2016). MIPs are synthesized by

mixing the template molecule with a functional monomer in an inert porogenic solvent, resulting in the formation of a pre-polymerisation complex. This complex polymerises in the presence of a cross-linking agent (that fixes the monomer around the template and allows the generation of the three-dimensional polymer network) and an initiator. After completion of the polymerisation process, the template is removed from the polymeric matrix leaving specific cavities whose shape, size and functional groups are complementary to the template molecule. Therefore, a molecular memory is generated inside the polymer, in which the target molecule is now able to rebind with a very high specificity. In this way, the MIP acquires the ability to selectively recognise the target molecule in the presence of other closely related molecules; a process that is very similar to the “lock and key” mechanism of enzymes (Chen et al., 2016; Madikizela et al., 2018a). A schematic summary of preparation of a MIP, signal enhancement strategies and detection mechanisms in electrochemical sensors is shown in Fig. 2.

The recognition properties of a MIP are compared with a non-imprinted polymer (NIP) that is prepared in the same conditions, but without the inclusion of the template molecule. Obviously, the great challenge is to obtain a MIP with the highest selectivity for the target molecule (Arrigo and Baroni, 2020).

The necessity of quantifying very low contaminant concentrations, the complexity of environmental samples and the limited availability of sensitive and selective methods are the principal difficulties for environmental monitoring (Madikizela et al., 2018b; Madikizela et al., 2018c; Namieśnik, 2000; Spietelun et al., 2013). In this context, suitable sample preparation techniques have been applied to selectively isolate and preconcentrate the analytes prior to their determination (Sarafraz-Yazdi et al., 2012). Many research papers have shown the use of MIPs as selective sorbents for extraction of various contaminants from complex sample matrices (Madikizela et al., 2018c). Despite the advantages of this advanced technical application of MIPs, they are also excellent tools to be used as recognition elements in electrochemical sensing (Li et al., 2019b). As mentioned before, when compared with biological receptors, such as antibodies or enzymes, MIPs are cheaper, more robust and have a greater reusability. However, the optimisation of the synthesis process to obtain a selective MIP is often a laborious and time-consuming task that not always leads to a suitable MIP as recognition element in a sensor. In this context, to avoid the extent and trial-and-error of experimental procedures, computational simulations have been employed to describe, predict and analyse molecular imprinting systems (Zhang et al., 2019). With a sustainable strategy,

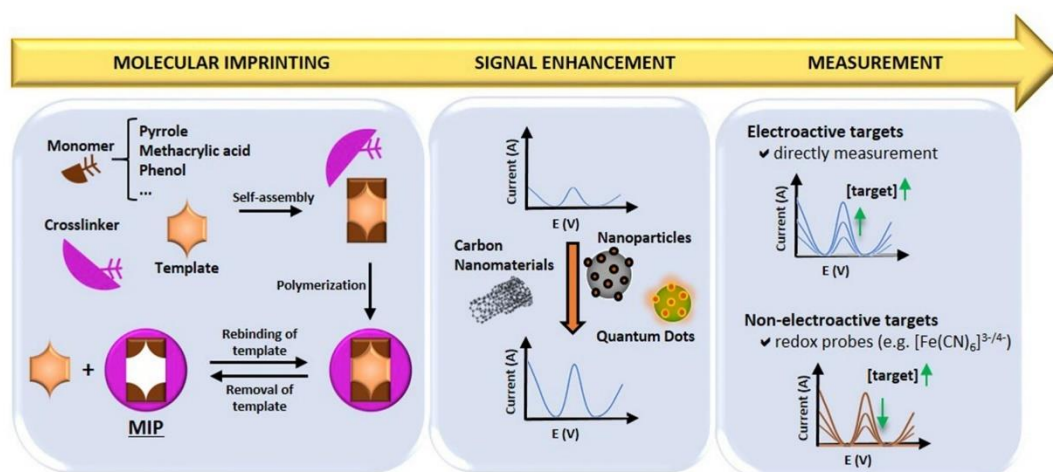


Fig. 2. Summary of the MIPs preparation procedure, signal enhancement and sensing mechanism of electrochemical MIP-based sensors.

various reports have shown that computational methods provide a strong guidance regarding on the design of MIPs (Khan et al., 2019; Rebelo et al., 2020; Xie et al., 2020).

Because of the differences in the templates' properties and the potential applications of the MIPs, a great variety of strategies for their preparation, such as bulk, suspension, emulsion and precipitation polymerisation, and surface imprinting, have extensively been reviewed (Ansari and Karimi, 2017b; Belbruno, 2019; Chen et al., 2016; Ertürk and Mattiasson, 2017; Lahcen and Amine, 2019; Mayes and Whitcombe, 2005). In the case of MIP-based electrochemical sensors, there are different approaches for the integration of the MIPs with the transducer (Gui et al., 2018, 2019; Toro et al., 2015). The main ones are i) the mixture of the MIP with the material of the working electrode before the preparation of the electrode; ii) the immobilisation (usually by drop coating) of a previously prepared MIP or MIP-based composite with conductive/nanomaterials on the transducer surface; and iii) electropolymerisation. These synthesis and integration strategies are reviewed in the next sub-section.

2.1. Preparation processes of MIPs

In the classical MIP preparation methods mentioned above, the bond between the template and the monomer can either be covalent or non-covalent. Non-covalent binding is more flexible since the removal and subsequent rebinding of the template is easier (Zaidi, 2017; Zhou et al., 2019). Some problems that still need to be overcome are related to the elution of polar compounds, which have a low solubility in organic solvents, the reduced number of effective recognition sites, the slow mass transfer rate and the incompatibility of MIPs with aqueous media (Chen et al., 2011). So, alternative imprinting routes or new imprinting techniques are constantly being developed.

As mentioned in the introduction, theoretically, MIPs can be synthesized for any analyte, but there is no specific strategy for a particular class of analytes; i.e. the optimisation of the imprinting process by experimental studies is crucial to produce the best MIP (or the MIP with the desired characteristics) for the target molecule. Bulk polymerisation is the conventional and most popular approach due to its simple operation and low production costs. In this method, also known as mass polymerisation, the template, functional monomer, cross-linker and initiator are mixed in a single reactor (Farooq et al., 2018). This process results in a rigid monolithic polymer matrix that, after crushing and grinding, may have some shortcomings such as the loss of binding sites that can lead to a low yield (Chen et al., 2011; Farooq et al., 2018). Although there are different protocols for the preparation of MIPs by bulk polymerisation, this method follows a typical sequence involving five main steps: pre-polymerisation of the functional monomers with the template molecules, addition of the cross-linking agents and initiators, polymerisation by heating for an extend period of time, extraction of the template and crushing the crosslinked mass into powder. Precipitation polymerisation, suspension polymerisation and emulsion polymerisation are three spherical MIP synthesis methods. In these methods, the polymerisation procedure is similar to bulk polymerisation, but the post-treatment steps are not required, which reduces the number of steps and, more importantly, the probability of destroying the imprinted cavities (Chen et al., 2011; Xiao et al., 2018). Precipitation polymerisation is carried out in a large volume of an organic solvent in which the polymers are insoluble and precipitate. This method is free of surfactants and allows to control the size of the particles, but their shape remains irregular. Suspension and emulsion polymerisation are used to prepare micro spherical MIPs and to increase monodispersity. For emulsion polymerisation an oil/water biphasic system is prepared and surfactants are added to the organic phase to prevent diffusion across the continuous aqueous phase (Wackerlig and Schirhagl, 2016). Compared to the precipitation strategy, suspension and emulsion polymerisation require more reagents and the purity and performance of the products are affected by the presence of surfactants remnants (Chen et al., 2016).

Another process for the preparation of MIPs is surface imprinting, in which electropolymerisation is frequently used. In *in-situ* electropolymerisation, using cyclic voltammetry (CV), the imprinted polymers are produced by coating surfaces at a well-controlled rate, producing an ultrathin polymeric film. In this process, the CV cycles and time can be used to determine and regulate the adhesion and the morphology of the polymeric film on the surface of substrates, facilitating the optimisation of the synthesis and providing highly reproducible MIPs in terms of binding capacities and immobilisation on the substrate's surface. Moreover, this technique facilitates template removal since the obtained MIP film is thin and all the imprinting cavities are situated on or closely at the surface (Cieplak and Kutner, 2016). So, electropolymerisation is usually a simple and fast procedure involving three steps: solubilization and interaction of a functional monomer with the desired template in a solvent, and electrochemical coating followed by extraction of the template (Crapnell et al., 2019). Other strategies for surface imprinting have also been studied. For example, the sol-gel process is an approach that can facilitate MIP preparation in aqueous matrices (Moein et al., 2019; Zhou et al., 2019). This technique consists of the use of silica-based materials as supports that allow the use of green solvents and an easy construction at room temperature without the problem of chemical or thermal decomposition observed in bulk polymerisation (Moein et al., 2019; Pohanka, 2017; Zhou et al., 2019). There are two main steps for sol-gel processing: sequential hydrolysis of silane in the presence of an acidic or basic catalyst and condensation of a series of silane monomers to form siloxane bonds. With time, a porous and a strong three-dimensional network is formed by colloidal particles aggregation (Mujahid et al., 2010; Yang et al., 2013). Sol-gel materials offer high thermal stability and porosity, which combined with molecular imprinting systems play a significant role in the performance of MIPs. The lack of functional monomers, the low sensitivity, slow diffusion kinetics and long response times are some drawbacks of this process (Adumitrăchioaie et al., 2018; Zhou et al., 2019).

Many materials for surface modification are also used in surface molecular imprinting processes such as Fe₃O₄ magnetic nanoparticles (Fe₃O₄ MNPs) and mesoporous SiO₂ nanoparticles (SiO₂ NPs) (Fig. 2). Besides a larger specific surface area, magnetic nanoparticles have superior advantages in the removal of solvents and in the fast and easy isolation of the analyte from samples by applying a magnetic field (Huang et al., 2018; Ye, 2013). SiO₂ NPs provide a stable solid support due to their mesoporous properties, allowing higher stability and sorption capacities in comparison with ordinary imprinting methods (Wang et al., 2011). By virtue of the controllable methods and ease of modification, surface imprinting technologies have been explored by several research groups. Furthermore, they have also included functional nanomaterials in the imprinted shell layers to improve the MIP-based sensors' electron transfer rates (Beluomini et al., 2019; Maduraiveeran et al., 2018). Carbon nanotubes (CNTs), graphene and quantum dots (QDs) have received special attention in the preparation of MIPs. Among the carbon materials, graphene is the most widely used because of its unique physicochemical properties. Besides its low electronic noise and thin 2-dimensional surface, graphene has strong interaction with carbon-based ring compounds, such as some contaminants (Zaidi, 2017). QDs are a kind of conducting nanocrystals that have excellent photoluminescence properties (Shi et al., 2019). QDs possess high luminescence efficiency and photostability, a broad absorption spectrum, narrow fluorescence emission bands and quantum-size effects (Nsibande and Forbes, 2019). The combination of their excellent properties with the specific recognition of MIPs is gaining more attention as an alternative for sensitive and selective detection of contaminants (Nsibande and Forbes, 2019; Sobiech et al., 2019; Wang et al., 2019; Zhou et al., 2018). Prior to molecular imprinting, QDs can be loaded on an electrode surface or incorporated in the surface of supporting nanomaterials such as SiO₂ NPs (Tan et al., 2016; Yola and Atar, 2017).

2.2. Functional monomers

The selection of an appropriate functional monomer is an essential step for the successful construction of MIPs with desired affinity and selectivity. Template-monomer interactions can be changed by the combination of different functional groups. So, many types of commercially available functional monomers have been studied, based on their potential to establish hydrogen bonds or electrostatic interactions with the template. Among the functional monomers reported in the literature, methacrylic acid (MAA), pyrrole (Py), and phenol (Ph) and derivatives are the most widely used in electrochemical MIP-based sensors for the detection of contaminants. Due its nature, MAA is the most popular carboxylic acid-based monomer used in bulk polymerisation. MAA not has only the ability to serve as both hydrogen bond donor or acceptor, but it can also interact with the template in various ways, including strong ion-pair and dipole-dipole interactions, and van der Waals forces, increasing its broad applicability (Mayes and Whitcombe, 2005). Py is a conductive (to some extent) and electropolymerisable monomer, well known to partially cross-link (Sadriu et al., 2020). Without the need to add a cross-linker, electropolymerisation is the most efficient method to synthesize PPy MIPs. The formation of the PPy film is fast, and a chemically and mechanically stable polymer is obtained (Sadriu et al., 2020). As a result of its good permeability, the use of PPy also allows easier extraction and rebinding of the template. Nevertheless, there are several experimental variables that can affect the chemical and physical properties of PPy formed on the electrode's surface, such as solvent, electrolyte, temperature and pH (Sadki et al., 2000). In contrast, Ph and p-aminothiophenol (PATP) are electronically non-conducting polymers which are also extensively used in electropolymerisation. The insulation properties of the formed polymer films can be improved by using self-assembled monolayers (Sharma et al., 2012); particularly PATP, which contains thiol groups, has been used for strong binding on gold surfaces. All these functional monomers can establish strong hydrogen bonds with templates, demonstrating that this effective interaction is a promising option to take into account during MIP preparations (Ansari, 2017).

2.3. Detection mechanisms

When using electrochemical MIP-based sensors, electrical signal (current or potential) changes, which are proportional to the concentration of the target analyte, are recorded (Li et al., 2012). Two principal techniques are used to detect the specific recognition events: voltammetry and electrochemical impedance spectroscopy (EIS) (Li et al., 2019b).

The voltammetric methods include linear sweep voltammetry (LSV), CV, differential pulse voltammetry (DPV) and square wave voltammetry (SWV). In these methods, the electroactivity of the analyte conditions the detection mechanism. For electroactive molecules, the current is measured directly, where the signal outputs ideally are the faradaic currents resulting from the oxidation/reduction of the analytes after binding in the MIP. In this case, under controlled conditions a correlation can be made between the concentration of the analyte and the measured current (Chen et al., 2016). When non-electroactive targets are involved, the signal can be produced using redox probes such as $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (Li et al., 2012). In this case, also called the "gate-controlled" mechanism, the current response of the redox probe is inversely proportional to the concentration of the analyte since higher concentrations lead to fewer channels for the diffusion of the redox probe to the electrode surface, and consequently to the decrease of the signal (Gui et al., 2019) (Fig. 2).

EIS is a powerful tool to investigate electron transfer and diffusion processes that occur at the electrode/electrolyte interface. The typical Nyquist plot obtained in EIS has two areas: at high frequencies a semi-circle portion corresponds to the electron transfer resistance, and at low frequencies diffusion limited process are observed by a linear

portion (Li et al., 2018). During the MIP's assembly process, changes in the surface properties of the electrode occur and different Nyquist plots are observed. Generally, the rebinding of the template in the cavities of the polymeric film results in an increase of the charge transfer resistance on the surface of the electrode, thus semi-circles with larger diameters are observed.

3. MIP-based electrochemical sensors for contaminant analysis

3.1. Pharmaceuticals

Pharmaceuticals are widespread micropollutants and are ubiquitous in waters and soils (Afonso-Olivares et al., 2017; Kamba et al., 2017). They have been released into the environment for decades through various ways, such as metabolic excretion in its original form or as metabolites, improper disposal of expired medication, and escaping the modest removal efficiencies of WWTPs (Björlenius et al., 2018; Rivera-Jaimes et al., 2018). Despite their low concentrations, their long half-lives amplifies effects from drug-drug interactions that can potentially be hazardous (Evgenidou et al., 2015; Kamba et al., 2017). Subtle effects of pharmaceutical compounds on aquatic and terrestrial organisms have been reported (Boxall, 2004) and, more recently, a major concern of the public and the scientific community includes bacterial resistance (Ayukekbong et al., 2017; Richardson and Ternes, 2014).

There is no definitive date that marks the emergence of the identification of pharmaceuticals in the environment (Daughton, 2016), but over the past three decades various methods and approaches have been developed to assess their profiles and occurrence patterns in different environmental compartments (Evgenidou et al., 2015; Yang et al., 2017). Undoubtedly, the knowledge of concentration levels of pharmaceuticals is essential as a starting point to apply more advanced treatments to improve their removal and, consequently, minimise their environmental risk (Afonso-Olivares et al., 2017).

Since 2009 several works have been dedicated to the development of electrochemical MIP-based sensors for the determination of a wide variety of pharmaceuticals including antibiotics, anti-inflammatories, analgesics, hormones, blood lipid regulators, β -blockers, antidepressants, antiepileptics and cytostatic drugs (Des Azevedo et al., 2013; Florea et al., 2015; Futra et al., 2016; Han et al., 2016; Miege et al., 2009; Özcan and Topcuogullari, 2017; Xiao et al., 2017; Yang et al., 2017). Many different strategies based on new imprinting techniques and polymerisation methods, and the use of innovative nanomaterials have been studied to improve the performance of the sensors (Adumitrăchioaie et al., 2018; Uzun and Turner, 2016). However, only a few electrochemical MIP-based sensors were applied to environmental samples. This is mainly because of the low levels of contaminants in the environment. Table 1 summarises the main characteristics of electrochemical MIP-based sensors for the detection of different classes of pharmaceuticals in water samples.

3.1.1. Antibiotics

Antibiotics are the most frequently studied group of pharmaceuticals for electrochemical detection using MIP-based sensors, showing the concern about their presence in waters and the importance of their detection.

Sulfamethoxazole (SMX) is one of the most frequently sulfonamide bacteriostatic antibiotic detected in the aquatic environment, soils and sediments due to its wide spectrum of applications and poor ability to be metabolised (Fekadu et al., 2019; Tao et al., 2019; Zhao et al., 2015). SMX has been detected in industrial effluents, in WWTP effluents and in surface water at concentrations between 0.036 nM and 7.2 nM with detection frequencies of more than 80% (García-Espinoza and Mijaylova Nacheva, 2019). In Africa, it was the most detected compound in water samples, reaching concentrations far higher than the ecotoxicity endpoints (Fekadu et al., 2019). Its presence revealed high risk to sensitive aquatic organisms (Zhang et al., 2012) with possible mutagenic activity

Table 1
Electrochemical MIP sensors constructed on different sensing platforms for the detection of pharmaceuticals in water samples.

Target	Functional monomer	Polymerisation method/ Transducer	Electrochemical technique	Samples	Linear Range (nM)	LOD (nM)	Reference
Sulfamethoxazole	Py	Electropolymerisation, BDDE	SWV	Lake water	1.0×10^2 – 1.0×10^5	24^a	Zhao et al. (2015)
	MAA	Bulk polymerisation, SPCE, Fe ₃ O ₄ MNPs	EIS	Seawater	0.1 – 1.0×10^7	1.0×10^{-3b}	Zamora-Gálvez et al. (2016)
Sulfanilamide	Py	Electropolymerisation, PGE	DPV	Ground water	50 – 1.1×10^3 1.1×10^3 – 4.8×10^4	20^c	Tadi et al. (2014)
Azithromycin	4-ABA	Electropolymerisation, SPCE	DPV	River water and tap water	5.0×10^2 – 1.0×10^4	80^d	Rebelo et al. (2020)
Erythromycin	m-PD	Electropolymerisation, SPANe	DPV	Tap water	2–16	0.1^b	Ayankajo et al. (2020)
Chloramphenicol	DAM	Bulk polymerisation, MWCNTs, AuNPs, GCE	DPV	Seawater and reservoir water	0.31×10^2 – 3.1×10^5	74^b	Zhao et al. (2012)
Ciprofloxacin	MAA	Bulk polymerisation, Ch-AuNPs, GCE	DPV	Mineral and tap water	1.0×10^2 – 1.0×10^5	2.1×10^{2e}	Surya et al. (2020)
Lomefloxacin	o-PD and β -CD	Electropolymerisation, Au electrode, Fe PC	DPV	River and lake water	1 – 1.2×10^2	0.2^f	Li et al. (2020a)
Chlortetracycline	o-PD	Electropolymerisation, GO, GCE	DPV	Tap water and laboratory wastewater	1.0×10^4 – 5.0×10^5	Not mentioned	Liu et al. (2013)
Cefalexin	I3AA	Electropolymerisation, GCE, BDDE	DPV	River water	10 – 1.0×10^3	3.2 and 4.9^b	Feier et al. (2019)
Mebendazole	MAA	Electropolymerisation, Fe-NCNF, GCE	DPV	Tap water and river water	10 – 1.5×10^3	4^a	Rao et al. (2018)
17- β -estradiol	MNA	Electropolymerisation, PtNPs, GCE	DPV	Hospital wastewater and tap water	30 – 5.0×10^4	16^a	Yuan et al. (2011)
	PATP	Electropolymerisation, AuNPs, Au electrode	LSV	River water	3.6×10^{-6} – 3.6	Not mentioned	Florea et al. (2015)
	An	Bulk polymerisation, Fe ₃ O ₄ MNPs, SPCE	SWV	River water	50 – 1.0×10^4	20^b	Lahcen et al. (2017)

4-ABA: 4-aminobenzoic acid; β -CD: β -cyclodextrin; An: aniline; AuNPs: gold nanoparticles; BDDE: boron doped diamond; Ch-AuNPs: chitosan gold nanoparticles; DAM: diethylaminoethyl methacrylate; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; Fe-NCNF: nitrogen-doped carbon nanosheet frameworks decorated with Fe; Fe-PC: Fe doped porous carbon; GCE: glassy carbon electrode; GO: graphene oxide; I3AA: indole-3-acetic acid; LSV: linear sweep voltammetry; m-PD: m-phenylenediamine; MAA: methacrylic acid; MNA: 6-mercaptopuric acid; MNPs: magnetic nanoparticles; MWCNTs: multiwalled carbon nanotubes; o-PD: o-phenylenediamine; PATP: p-aminothiophenol; PGE: pencil graphite electrode; PtNPs: platinum nanoparticles; Py: pyrrole; SPANe: screen printed gold electrode; SPCE: screen printed carbon electrode; SWV: square wave voltammetry.

^a LOD calculated as $S/N = 3$.

^b LOD calculated as 3 times the standard deviation of the blank divided by the slope.

^c LOD calculated as 3.3 times the standard deviation of the blank divided by the sensitivity.

^d LOD calculated as 3 times the standard deviation of the intercept divided by the slope.

^e LOD formula not specified

and the potential to disrupt the endocrine system (Archer et al., 2017; Zhao et al., 2015). Therefore, its monitoring is relevant to guarantee the health of all organisms. In 2015, the first electrochemical MIP-sensor, based on a PPY modified Boron Doped Diamond Electrode (BDDE), was developed for the determination of SMX in spiked lake water (Zhao et al., 2015). This sensor was prepared by electropolymerisation of Py and the determination of SMX was achieved through its direct oxidation using SWV. A good sensitivity, a limit of detection (LOD) of 24 nM and minimal interferences of structurally similar sulfonamides (sulfadimethoxine, sulfadiazine and sulfafurazole) were observed. Although BDDEs have excellent properties, some researchers avoid their use because of the very heterogeneous composition of the BDDE's surface (Feier et al., 2019) and because of the possibility of its degradation in alkaline solutions (Luong et al., 2009). Sulfanilamide (SN) is another member of the sulfonamide family and has mainly been detected in surface waters, with high detection rates in Chinese rivers in comparison with other countries. Tadi et al. (2014) also used electropolymerisation of Py for the fabrication of a sensor for the analysis of SN. In this case, a pencil graphite electrode (PGE) was used. Py was selected as the best functional monomer compared with furan, thiophene, methylthiophene and methylpyrrole since computational studies showed it has the highest binding

interaction energy with SN. DPV was used for its direct analysis (oxidation), achieving an LOD of 20 nM. This sensor showed good selectivity towards SN; species with analogue structures such as SMX, sulfathiazole and sulfadiazine did not significantly interfere in the analysis. The applicability of this sensor was tested in spiked human serum and ground water samples, obtaining recoveries between 98 and 115%.

The use of nanomaterials is one of the most widely used strategies to increase the electrode's surface area and consequently the sensitivity of the method. Zamora-Gálvez et al. (2016) reported a specific and highly sensitive composite-based sensing system, based on MIP-modified Fe₃O₄ MNPs, to detect SMX via EIS (Fig. 3A). In this work, nano-sized MIP cavities were constructed on the surface of the Fe₃O₄ nanoparticles by bulk polymerisation using MAA as the functional monomer. Because of the superparamagnetic properties of the formed composite, this sensing system allowed easy separation, pre-concentration and manipulation of the target analyte. After magnetic deposition of the composite on the surface of a screen printed carbon electrode (SPCE), the electron transfer resistance (R_{ct}) was monitored by EIS in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$, which increased with increasing SMX concentrations. This sensing system showed an excellent LOD of 1×10^{-3} nM and was applied to spiked

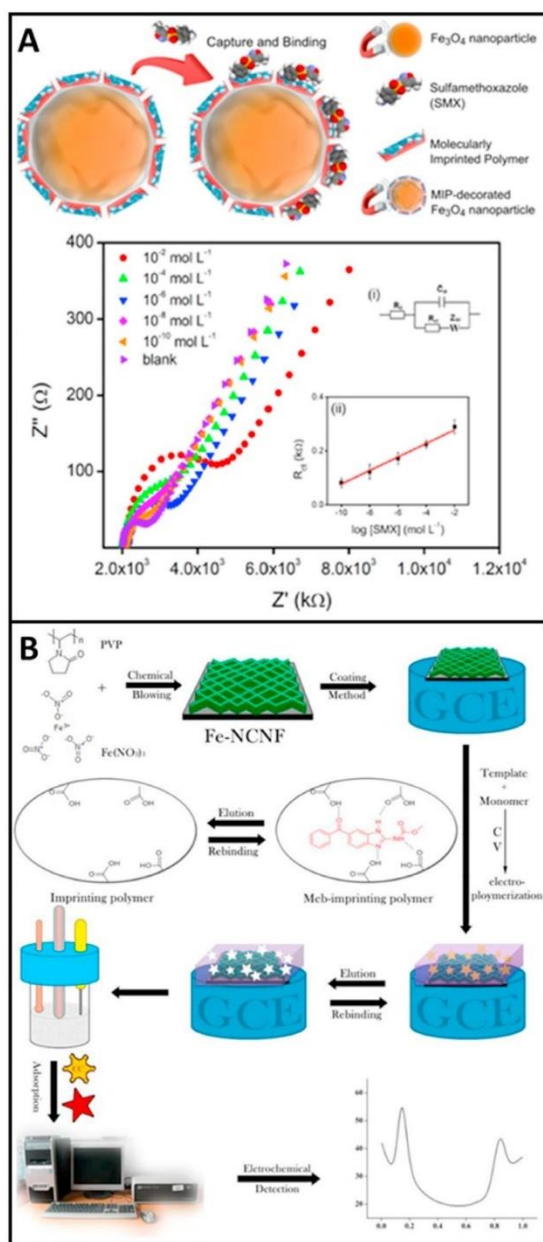


Fig. 3. A) Schematic representation of the MIP-based sensor for SMX detection based on MIP-modified Fe_3O_4 MNPs and the Nyquist plots and calibration curve for the determination of SMX. Reproduced and adapted with permission from Zamora-Gálvez et al. (2016). Copyright 2016 American Chemical Society. B) Preparation and analysis procedures of the Fe-NCNF/MIP/GCE for detection of MB. Reproduced from Rao et al. (2018) with permission from Elsevier.

seawater samples, achieving recoveries between 87 and 106%. In comparison with the previously described SMX sensor (Zhao et al., 2015), and although higher amounts of reagents were required, a much lower LOD was obtained. In this work MIP-modified Fe_3O_4 MNPs were

used to separate and pre-concentrate SMX and their magnetic deposition onto the surface of SPCEs offered an innovative design in the field of disposable sensors.

Azithromycin (AZY) and erythromycin (ERY) are frequently prescribed macrolide antibiotics to treat many different bacterial infections. Due to their wide use, the difficulty of removing them by common wastewater treatments and their frequent detection in water bodies, AZY and ERY were included in the EU Watch List (Decision, 2018/840/EU) of substances that could pose a significant risk to aquatic environments (Ayankojo et al., 2020; Rebelo et al., 2020). Recently, a low-cost and user-friendly electrochemical MIP sensor to detect AZY in water was reported (Rebelo et al., 2020). By a computational study based on density functional theory (DFT), 4-aminobenzoic acid (4-ABA) was chosen as the most suitable monomer, which was electropolymerised on the surface of a SPCE. The analysis of AZY was performed by its oxidation using DPV (LOD = 80 nM) and the sensor displayed great recognition behaviour in the presence of other interfering compounds. It was successfully applied to the analysis of spiked tap water and water samples collected upstream of a WWTP output in the Ave river (Portugal). A similar system was used by Ayankojo et al. (2020) to obtain the first ERY-selective MIP film integrated with a screen printed gold electrode (SPAUE). In their work, the MIP was generated directly on the SPAUE via electropolymerisation of m-phenylenediamine (m-PD). DPV measurements performed using a $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe, allowed the determination ERY with an LOD of 0.1 nM.

Chloramphenicol (CAP), one of the oldest antibiotics, is widely used in veterinary medicine and to promote the growth of food-producing animals. This antibiotic was completely banned for use in food-producing animals in many European countries due to its potential serious adverse effects (Zhao et al., 2012). Despite legal bans, CAP is easily available and is still used because of economic interests (Ding et al., 2017; Shaheen et al., 2017). Zhao et al. (2012) reported the first electrochemical sensor for the determination of CAP in natural water samples, combining MIPs and the advantages of carbon nanotubes (CNTs) and gold nanoparticles (AuNPs). The functional monomer, diethylaminoethyl methacrylate (DMA), was added together with CAP to perform bulk polymerisation and the direct detection process was based on DPV using a glassy carbon electrode (GCE). The LOD of this sensor was 74 nM and the selectivity for CAP detection was demonstrated in the presence of penicillin-G, thiamphenicol and their analogue p-nitrophenol. Bulk polymerisation was also used to construct a novel sensor modified with chitosan and AuNPs (Ch-AuNPs) to detect ciprofloxacin (CIP), an antibiotic that has been detected in surface waters. Ch-AuNPs were used as the supporting material to polymerize MAA in the presence of CIP (Surya et al., 2020). The resulting MIP was drop casted onto the surface of a GCE and its capability to quantify the antibiotic in real samples was tested using mineral and tap water. Another antibiotic of the quinolone family that has also been detected in surface waters is lomefloxacin (LFX). Li et al. (2020a) developed an effective MIP sensor for its determination in river waters. They used a gold electrode modified with Fe-doped porous carbon (Fe-PC) and electropolymerisation to produce the MIP film. Because of the hydrogen bonds and host-guest inclusion interactions, o-phenylenediamine (o-PD) and β -cyclodextrin (β -CD) were chosen as functional monomers. This methodology allowed high selectivity towards LFX, because of the use of the MIP prepared with bifunctional monomers, and high sensitivity, which was attributed to the large specific surface area, rich porosity, and good catalytic property of Fe-PC. An indirect detection approach and DPV were used for the analysis of LFX detection in river waters, achieving an LOD of 0.2 nM.

Tetracycline antibiotics are highly prescribed worldwide because of their favourable properties both for human and animal therapy (Borghi and Palma, 2014; Li et al., 2020b; Van et al., 2020). More than 70% of these antibiotics are released in their active forms into the environment (Daghbir and Drogui, 2013; Javid et al., 2016) and, due its presence in waters, several studies have shown direct effects on microbial

community structures (Grenni et al., 2018). Liu et al. (2013) developed a sensor to detect chlortetracycline (CTC), a derivate of tetracycline, based on electropolymerisation of o-PD and controlled electrochemical reduction of graphene oxide (GO) on a GCE. DPV measurements evaluated the electrochemical performance of the MIP-sensor for the analysis of tap water and a spiked laboratory wastewater sample. Although a current amplification effect and a higher electroconductivity through the electrochemical reduction of GO was expected, the sensor did not show a high sensitivity and the authors acknowledge the need to expand the linear concentration range ($1.0 \times 10^4 - 5.0 \times 10^5$ nM).

Cefalexin (CFX) is a broad-spectrum antibiotic that belongs to the class of cephalosporins (Rahim et al., 2019). Cephalosporins have been found in different aqueous matrices and river sediments in which cefalexin is the most frequently detected compound (Ribeiro et al., 2018). Its ubiquitous presence in sewage and wastewater is correlated with human and livestock populations. An electrochemical MIP sensor for its determination was recently described by Feier et al. (2019). Two different electrodes, GCE and BDDE, were used to assess the performances of the MIP sensors. Indole-3-acetic acid (IAAA) was chosen as the functional monomer, not only because it can be electropolymerised in aqueous solution, but also because of its functional groups that can form strong interactions with CFX. The indirect quantification of CFX was carried out by using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and DPV. The LOD obtained with the BDDE was lower, but its precision was worse compared to the GCE. As mentioned above for the MIP sensor proposed for SMX (Zhao et al., 2015), BDDE provided very attractive advantages which was confirmed in this work: a more pronounced surface imprinting process and, consequently, a better sensitivity was achieved. However, GCE offered a better reproducibility and showed to be the most reliable transducer. This MIP sensor was easy to prepare and was successfully applied to river water analysis.

3.1.2. Anthelmintic

Unlike the variety of MIP-sensors for antibiotic analysis, to the best of our knowledge only one MIP-based sensor for the detection of anthelmintic pharmaceuticals has been reported. This sensor combined hybrid nanomaterials with a MIP prepared by electropolymerisation for the simultaneous determination of mebendazole (MB) and catechol (Rao et al., 2018). A carbon nanosheet was created through a chemical blowing process on a GCE (Fig. 3B) and after electrochemical polymerisation of MAA with MB, it was observed that, due to their thin shells, nitrogen-doped carbon nanosheet frameworks decorated with Fe (Fe-NCNF) played a significant role in absorbing more MB molecules. Consequently, its frame structure with a large specific surface area allowed a good electron-transfer for the oxidation of MB at 0.82 V, but also supported the strong adsorption of catechol, which was electrochemically oxidised at 0.15 V. An easy-to-prepare and efficient MB sensor was obtained in this work. Fe-NCNF was synthesized using a simple process and its combination with a MIP, synthesized through electropolymerisation, amplified the electrochemical response of the sensor.

3.1.3. Hormones

Other substances present in environmental waters at concentrations of toxicological and carcinogenic concern are hormones, especially 17- β -estradiol (E2). E2 is commonly used in contraceptive pills and its release in water has gained notable attention, mainly because it is considered the major contributor to endocrine disruption of many species in the ecosystem (Salste et al., 2007). Additionally, it has been linked with breast cancer in women and prostate cancer in man (Adeel et al., 2017). E2 was also added to the EU Watch list of emerging aquatic contaminants (Decision, 2018/840/EU), highlighting the importance of developing sensitive and accurate methods for its determination. In 2011, Yuan et al. (2011) reported an electrochemical MIP-based sensor for E2 quantification using a GCE modified with platinum nanoparticles (PtNPs). The MIP for E2 was constructed through the

electropolymerisation of 6-mercaptosuccinic acid (MNA) and DPV was applied to directly detect the E2 binding to the MIP since it is an electroactive species. This sensor reached an LOD of 16 nM and was successfully applied to spiked hospital wastewater and tap water analysis. Later, Florea et al. (2015) developed another MIP sensor able to detect E2 via electropolymerisation of PATP. Assembled AuNPs on the surface of an Au electrode were used as electron wire for signal amplification, but in this work the response of the MIP sensor to E2 was measured indirectly by LSV. The combination of Fe_3O_4 with molecularly imprinting again showed to be an excellent tool for the highly specific detection of E2 (Lahcen et al., 2017). Lahcen et al. (2017) developed a Fe_3O_4 -MIP sensing system with high selectivity towards E2 for the analysis of river water samples. Following the traditional bulk polymerisation strategy, MIP-modified Fe_3O_4 MNPs were obtained and then used to modify the working electrode surface of SPCE by drop-casting, leading to the enhancement of the direct oxidation current obtained by SWV. The developed sensor achieved an LOD of 20 nM.

3.2. Pesticides

Over the past 50 years, global sales of pesticides have increased because their importance in agricultural production. They inhibit and prevent the growth of harmful animals, insects, invasive plants, weeds, and fungi. However, repeated applications result in their accumulation in soils and they can be transported to the aquatic environment by surface runoff (Rousis et al., 2017). Considering their chemical properties and persistence, the biodiversity and the ecosystems' health is jeopardised since their toxic action is not restricted to target pests (Carvalho, 2017). Among all available pesticides, herbicides, insecticides, and fungicides are the most used types. Although electrochemical detection of pharmaceuticals in water samples using MIPs appears an unexplored area, many publications have been reported about their use for the trace analysis of pesticides, which are summarised in Table 2.

3.2.1. Insecticides

Organophosphates persist for days/weeks in the aquatic environment and studies showed that they are accumulated by crustaceans and fishes (Carvalho, 2017). These are the insecticides for which most electrochemical MIP sensors have been developed. As shown in Table 2 different strategies were adopted for the detection of chlorpyrifos (CPF) and the use of GCE is common to all of them. Xie et al. (2010b) reported a molecular self-assembly strategy for electropolymerisation of PATP on the surface of AuNPs-modified GCE by the formation of Au-S bonds. A linear range between 5.0×10^2 nM and 1.0×10^4 nM and an LOD of 330 nM were obtained. This sensor showed good interclass selectivity and the authors tested its applicability in spiked tap water. A better LOD (4.08 nM), was attained via simple bulk polymerisation of MAA (Xu et al., 2017). A GCE was coated with a suspension of the resulting MIP and the binding of CPF was evaluated by DPV using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as redox probe. This simple sensor provided a good selectivity and its applicability was assessed in river water samples. However, an electrochemical MIP-sensor based on carbon nitride nanotubes (C_3N_4 NTs) decorated with graphene quantum dots (GQDs) (Yola and Atar, 2017) provided a much lower LOD (2.0×10^{-3} nM) in comparison with the previous works. As mentioned above, these nanomaterials have attracted great attention, and this work is one of the first reports that combines MIP with their excellent properties for application in wastewater samples. After the synthesis of the C_3N_4 NTs@GQDs composite by hydrothermal treatment, a suspension of this nanohybrid material was dropped onto the GCE surface. The electropolymerisation was performed using Py as functional monomer creating electrostatic interactions and hydrogen bonds with CPF. The performance of the prepared MIP sensor was evaluated directly by SWV. High conductivity of the electrode surface was achieved, and it was demonstrated that the introduction of a finite bandgap into graphene improved its gapless

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Table 2
Electrochemical MIP sensors constructed on different sensing platforms for the detection of pesticides in water samples.

Target	Functional monomer	Polymerisation method/ Transducer	Electrochemical techniques	Samples	Linear Range (nM)	LOD (nM)	Reference
Chlorpyrifos	PATP	Electropolymerisation, GCE, AuNPs	CV	Tap water	5.0×10^2 – 1.0×10^4	3.3×10^{2a}	Xie et al. (2010b)
	MAA	Bulk polymerisation, GCE	DPV	River water	0.1 – 1.0×10^4	4.1^b	Xu et al. (2017)
	Py	Electropolymerisation, GCE, C_3N_4 NTs, GQDs	SWV	Industrial wastewater	1.0×10^{-2} – 1	2.0×10^{-3c}	Yola and Atar (2017)
Methyl-parathion	PATP	Electropolymerisation, GCE, AuNPs, CNTs	LSSV	Distilled water and tap water	0.38 – 4.2	0.30^d	Wu et al. (2014)
	Ph	Electropolymerisation, SPuE, N-GS	CV	River water	3.8×10^2 – 3.8×10^4	38^d	Xue et al. (2014)
Parathion	MAA	Bulk polymerisation, CPE	SWV	Tap, river and lake water	1.7 – 9.0×10^2	0.5^d	Alizadeh (2009)
Diazinon	MAA	Bulk polymerisation, CPE	SWV	Well water	2.5 – 1.0×10^2	0.79^b	Motaharian et al. (2016)
	MAA	Bulk polymerisation, CPE, MWNTs	SWV	Tap and river water	1.0×10^2 – 2.0×10^3	0.13^b	Khadem et al. (2017)
Phosalone	APTES	Sol-gel method, CPME, Pt-UiO-66	SWV	Lake water and soil	0.50 – 2.0×10^4	0.078^b	Xu et al. (2020b)
Cypermethrin	Ph	Electropolymerisation, GCE, Fe@AuNPs, 2D-hBN	DPV	Wastewater	1.0×10^{-3} – 10	3.0×10^{-5d}	Atar and Yola (2018)
	DA and RC	Electropolymerisation, GCE, Ag–N@ZnO, CHAC	EIS	Tap water and soil	2.0×10^{-4} – 8	6.7×10^{-5e}	Li et al. (2019d)
Cyfluthrin	APTES	Sol-gel method, QDs, Nafion-MWCNTs	ECL	Seawater	0.46 – 2.3×10^2	0.12^a	Li et al. (2020a)
Triazophos	PATP	Electropolymerisation, Au-electrode, luminol	ECL	Tap water, reservoir water and river water	0.1 – 1.0×10^3	0.058^d	Li et al. (2019a)
Glyphosate	Py	Electropolymerisation, Au-electrode	DPV	Tap water	30 – 4.7×10^3	1.6^b	Zhang et al. (2017)
	MAC	Bulk polymerisation, PGE, AuNPs, MWCNTs	DPASV	Soil	49 – 4.7×10^2	2.1^f	Prasad et al. (2014)
	PATP	Electropolymerisation, Au-electrode, AuNPs	LSV	Tap water	5.9×10^{-6} – 5.9×10^{-6a}	4.7×10^{-6a}	Do et al. (2015)
2,4-dichlorophenol	MAA	Bulk polymerisation, GCE, microgel suspension, Ch, Nafion	DPV	Tap, river and drinking water	5.0×10^3 – 1.0×10^5	1.6×10^{3a}	Zhang et al. (2013)
	MAA	Bulk polymerisation, GCE, GO	DPV	Lake water	4 – 1.0×10^4	0.5^g	Liang et al. (2017)
	EDOT	Electropolymerisation, CFP	DPV	Lake, river and tap water	0.21 – 3.0×10^2	0.07^g	Maria et al. (2020)
4-Chlorophenol	o PD	Electropolymerisation, GCE, ZnO NPs	SWV	Wastewater	2.0×10^2 – 1.7×10^5	40^d	AL-Anmari et al. (2019)
Atrazine	Ph	Electropolymerisation, GCE, PtNPs, C_3N_4 NTs	SWV	Wastewater	1.0×10^{-3} – 1.0×10^{-1}	1.5×10^{-4}	Yola and Atar (2017)
2,4-Dichlorophenoxy acetic acid	Py	Electropolymerisation, GCE	CV	Tap water	1.0×10^{-3} – 1.0×10^4	8.3×10^{2a}	Xie et al. (2010a)
Paraquat	Py	Electropolymerisation, PGE, EBB	DPV	Dam water	5.0×10^3 – 5.0×10^4	2.2×10^{2a}	Sayyahmanesh et al. (2016)
Diuron	MAA	Bulk polymerisation, CPE, MWCNTs	SWV	River water	52 – 1.3×10^3	9.0^b	Wong et al. (2015)
Chloridazon	2-VP and MAA	Bulk polymerisation, CPE, MWCNTs	DPV	Ground, surface, seawater and drinking water	5.0×10^2 – 4.0×10^5	62^d	Ghorbani et al. (2020)
Hexazinone	2-VP	Bulk polymerisation, CPE	DPV	River water	0.019 – 0.11	2.6×10^{-3}	Toro et al. (2015)
Dicloran	MAA	Bulk polymerisation, CPE, MWCNTs	SWV	Tap water and river water	1.0×10^3 – 1	0.48^d	Khadem et al. (2016)
Tributyltin	APTES	Sol-gel method, SPCE, Fe_3O_4 MNPs	EIS	Sea water	5.0×10^{-3} – 5.0×10^3	5.4×10^{-3}	Zamora-Gálvez et al. (2017)

^a LOD calculated as 3 times the standard deviation of the blank.

^b LOD calculated as 3 times the standard deviation of the blank divided by the slope.

^c LOD calculated as 3.3 times the standard deviation of the intercept divided by the slope.

^d LOD formula no specified.

^e LOD calculated as S/N = 3.

^f LOD based on the minimal distinguishable signal for lower concentration of analyte.

^g LOD calculated as 3 times the standard deviation of the lowest concentration (or any concentration) divided by the slope.

nature, showing the potential role of GQDs in the development of electrochemical sensors.

In another example of the use of nanomaterials, electrochemical MIP sensors for the analysis of methyl-parathion (MP) were prepared using both electro- and bulk polymerisation. Wu et al. (2014) constructed a sensor based on AuNPs decorated with CNTs. After electrodeposition of

functionalised AuNPs on a MWCNTs/GCE surface, the electrode was immersed in a solution containing PATP. Since MP is electroactive, its recognition by the MIP could be directly monitored by LSV. The proposed sensor allowed a low LOD (0.30 nM) and was successfully applied to the determination of MP in spiked distilled and tap water, and apple and cucumber samples with recoveries ranging from 95 to 106%.

Another quantitative method for MP was based on nitrogen-doped graphene sheets (N-GS) and a MIP synthesized by electropolymerisation of Ph (Xue et al., 2014). Although N-GS has been described as an excellent sensing material for molecular imprinting, the developed MIP-sensor showed a worse analytical performance than the one described above (Wu et al., 2014) (see Table 2).

The use of carbon paste-based electrodes (CPE) has some benefits that include easy surface modification and very low ohmic resistance (Toro et al., 2015; Vytyas et al., 2009). These advantages were used to construct MIP sensors for parathion (Alizadeh, 2009) and diazinon (Motaharian et al., 2016), where the MIPs were synthesized by bulk polymerisation using MAA as functional monomer. The detection of these organophosphorus pesticides was performed by SWV and the authors demonstrated that the optimisation of the composition of the CPE is very important to improve the sensor's sensitivity.

The introduction of nanomaterials into CPEs seems to be crucial to

achieve the best performance. In the past few years the rapid growth of research interest in metal organic frameworks (MOFs) linked via the self-assembly of transition metal ions/clusters and organic ligands has been observed (Xu et al., 2020b). Very recently, Xu et al. (2020b) based on its favourable characteristics like highly ordered structure and exposed sites, prepared a novel disposable carbon paste microelectrode (CPME) MIP sensor on zirconium (Zr) based MOF (UiO-66) to detect phosalone (PAS). UiO-66 was combined with Pt nanoparticles and the MIP imprinted with PAS was produced by the sol-gel method with 3-aminopropyltriethoxysilane (APTES) as the functional monomer. The resulting sensor exhibited an LOD of 0.078 nM and its feasibility was evaluated in lake water and soil samples.

Cypermethrin (CYP) belongs to the class of pyrethroid insecticides and its insecticidal action is more effective and less toxic compared to the organophosphates. So, this pesticide has been widely used through the world. Recently, a MIP-based sensor to determine CYP in spiked

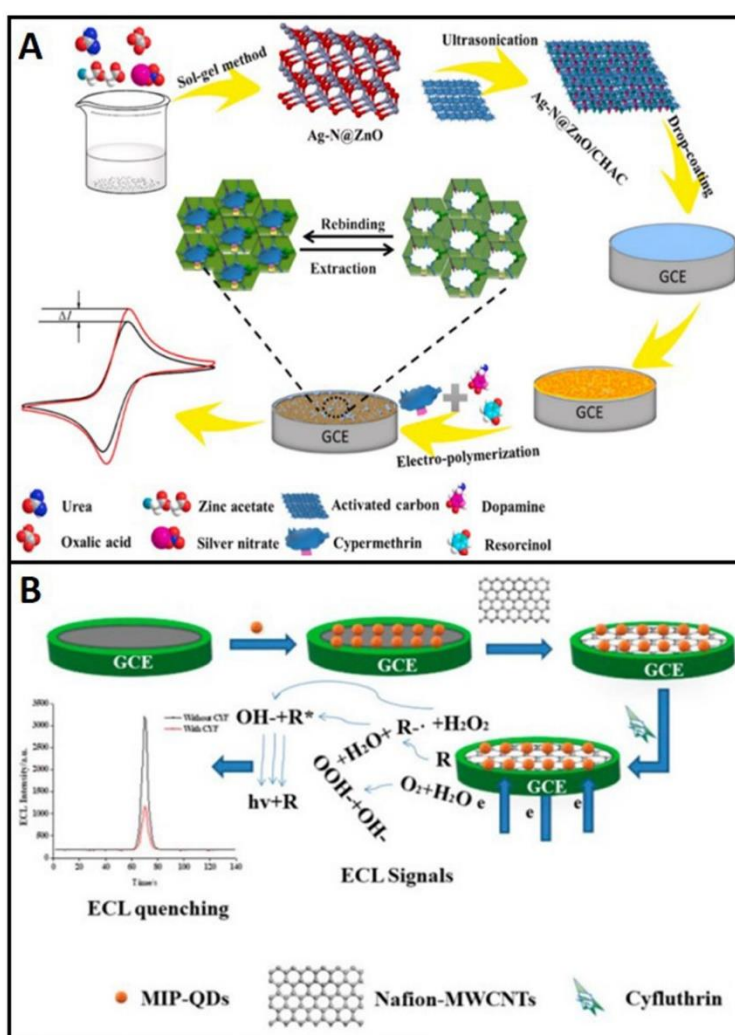


Fig. 4. A) Schematic representation of the fabrication and characterisation of a dual MIP monomer based on Ag-N@ZnO/CHAC/GCE. Reproduced from Li et al. (2019d) with permission from Elsevier. B) Schematic representation of a MIP ECL sensor to detect CYF. Reproduced from Xu et al. (2020a).

wastewater samples was reported (Atar and Yola, 2018). The sensing phase of the sensor was constructed on the surface of core-shell type nanoparticles (Fe@AuNPs) incorporating two-dimensional hexagonal boron nitride (2D-hBN) nanosheets through polymerisation of Ph. The highly sensitive properties of the prepared nanocomposite allowed a superior ability of specifically binding CYP and an extraordinary LOD (3.0×10^{-5} nM).

More recently, based on studies which showed a better performance of MIPs prepared with two or more functional monomers, Li et al. (2019d) produced a dual-monomer MIP for the analysis of CYP. Here, the preparation of the MIP sensor involved specific steps to produce a hybrid material by combining Ag and N co-doped zinc oxide (Ag-N@ZnO) with activated carbon (AC) (Fig. 4A). To produce an enlarged sensing surface and, consequently, amplify the signal, AC was chosen as a sensitising material on a GCE surface due to its low cost and easy availability. However, the authors reveal that the sole use of AC hinders the formation of a rigid layer on the electrode surface. This drawback was overcome by the addition of ZnO for better immobilisation. ZnO can easily be combined with other materials and doping can enhance its electrical conductivity. Therefore, as can be seen in Fig. 4A, Ag-N@ZnO was firstly produced by the sol-gel method and was then ultrasonically mixed with AC. After that, a suspension of the prepared solution was dropped on the GCE surface and the MIP was electropolymerised with dopamine (DA) and resorcinol (RC) as dual functional monomers. The electrochemical performance was evaluated by CV using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as redox probe and the sensor was applied not only to water samples, but also to a soil sample. Although this dual monomer MIP provided a novel strategy to enhance binding and affinity with target analytes, the LOD was not as low as the MIP sensor proposed by Atar and Yola (2018), in which only one functional monomer was used.

Electrochemiluminescence (ECL) combined with MIPs can improve the sensitivity and selectivity of the assay. The use of ECL has seen an exponential growth in electrochemical sensors. However, the use of ECL as detection technique for MIP sensors with applications in real water samples is still scarce. An example of this combination is the sensor developed by Xu et al. (2020a) to detect the insecticide cyfluthrin (CYF). They developed a MIP platform based on QDs as luminophore in the presence of H_2O_2 as co-reactant. In the ECL process, both the luminophore and the co-reactant are oxidised or reduced at the electrode forming radical species. Then, an electrochemical reaction between their redox products occurs and radiation is emitted. Due to its high emission quantum yield, luminol is a classic ECL luminophore. However, an increase in the demand of other alternatives has been observed because of limitations during its ECL process Li et al. (2019a). So, in the work developed by Xu et al. (2020a), the combination of a MIP with ECL based on QDs demonstrated high selectivity, good stability and controllability, where MWCNTs were utilised to improve the electrocatalytic activity and minimise surface fouling on the GCE. To simplify the electrode preparation process and improve the electrocatalytic activity, MIP-QDs were firstly synthesized by the sol-gel method using APTES as functional monomer. Then, the resulting MIP-QDs were used to coat the surface of a GCE (Fig. 4B). After drying, MWCNTs prepared in a Nafion solution, which can facilitate the adhesion and the ionic transportation across the electrode, were placed on the GCE surface. In the presence of CYF, the ECL and the redox peak current intensity decreased, which was accompanied by the increase of the electron transfer resistance. The LOD was 0.12 nM and good recoveries in seawater samples were obtained.

3.2.2. Herbicides

Glyphosate (Gly) is among the mostly widely used herbicides by farmers during the past 40 years. Although not proven, Gly has been associated with cancer in humans and a strong debate about its potential harmfulness has been generated (Silva et al., 2018). Due to its persistence in seawater (Mercurio et al., 2014), the need to identify trace levels of Gly in drinking water is also urgent. To the best of our

acknowledge, the most recent electrochemical MIP-sensor used for Gly detection in water samples was reported by Zhang et al. (2017). In this work, a simple electropolymerisation procedure, using Py as functional monomer, on an Au electrode was performed. The sensor presented good binding kinetics to Gly and showed good stability, selectivity, and sensitivity, and an LOD of 1.60 nM. The analytical signal was based on the use of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe and DPV. This MIP sensor exhibited a higher sensitivity when compared with the MIP sensor constructed by Prasad et al. (2014), where N-methacryloyl-L-cysteine (MAC) molecules were used as monomer in the bulk polymerisation process. However, the best sensitivity to detect GLY in water samples was obtained by the construction of an electrochemical sensor based on MIP-MOF films formed on Au surfaces through electropolymerisation of PATP functionalised with AuNPs (Do et al., 2015). A low LOD of 4.7×10^{-6} nM was achieved and the sensing capacity of the sensor was evaluated in tap water.

All other published MIP-sensors to detect herbicides with applications in water samples were based on the modification of GCE (AL-Ammari et al., 2019; Liang et al., 2017; Xie et al., 2010a; Yola and Atar, 2017; Zhang et al., 2013), PGE (Sayyahmanesh et al., 2016) or CPE (Ghorbani et al., 2020; Khadem et al., 2017; Toro et al., 2015; Wong et al., 2015). Additionally, a newly developed carbon fibre paper used as working electrode was also reported (Maria et al., 2020). In order to enhance the selectivity and precision, various nanomaterials were combined in these sensors.

As can be seen in Table 2, the LOD for 2,4-dichlorophenol (2,4-DCP), a typical chlorophenol that is widely employed in the synthesis of herbicides and insecticides, was much lower in the presence of GO (Liang et al., 2017). Moreover, Liang et al. (2017), using the same functional monomer (MAA), proposed a very simple methodology for the fabrication of a 2,4-DCP-MIP electrochemical sensor in comparison with Zhang et al. (2013). Besides using MAA as functional monomer, Zhang et al. (2013) also used a co-monomer, chlorohemin, to introduce chemically active sites into the MIP as well as a combination of chitosan (Ch) and Nafion for immobilisation of the MIP and to increase conductivity, respectively. This assembly probably blocked the mass transport to the electrode surface which resulted in a worse LOD. More recently, one of the first reports using a poly(3,4-ethylenedioxythiophene) (PEDOT) based MIP on carbon fibre paper, and the first applied to water samples, was published by Maria et al. (2020). To detect 2,4-DCP, carbon fibre paper was chosen as working electrode because of its porosity, offering abundant reaction sites. This novelty and the use of PEDOT as conductive polymer not only improved the LOD (0.07 nM), but also the stability and reproducibility. Other approach was presented by AL-Ammari et al. (2019), in this case, to explore the detection of 4-chlorophenol (4-CP). The MIP was prepared by electropolymerisation of o-PD with multifunctional nanomaterials, zinc oxide nanoparticles (ZnO NPs) and graphene platelets, on a GCE. Compared with other techniques applied for the detection of 4-CP, this MIP, which was tested in real wastewater samples, showed higher sensitivity.

Another excellent approach to improve the LOD was based on combining Pt NPs and C_3N_4 NTs, which were obtained by hydrothermal treatment and allowed minimised waste formation. Yola and Atar (2017) employed these nanomaterials to modify a GCE surface and, through electropolymerisation of Ph, they created a sensor that showed an LOD of 1.5×10^{-4} nM for the analysis of atrazine (ATR). PATP was also investigated for the determination of 2,4-dichlorophenoxy acetic acid (2,4-D) (Xie et al., 2010a). In this work, a simple procedure which involved electropolymerisation on the surface of a GCE, was developed. The 2,4-D molecules were removed from the polymeric matrix without solvent extraction, which is in accordance with “green chemistry”. However, this simple sensor did not show a great LOD.

Due to the favourable features of Py in aqueous media, it was chosen again as functional monomer for the recognition of paraquat in dam water (Sayyahmanesh et al., 2016). The electropolymerisation of the MIP occurred on top of a PGE in the presence of a functional doping ion

(eriochrome blue-black B (EBB)). EBB is an anionic complexing agent, which was used to maintain the electroneutrality during the reduction of Py and, consequently, to conserve its electroactivity. In order to enhance the electrochemical signal of diuron, a CPE modified with a MIP and MWCNTs was proposed by Wong et al. (2015). The MIP was synthesized via bulk polymerisation. This polymerisation technique was also adopted by (Ghorbani et al., 2020) for the determination of chloridazon (CLZ) in surface, ground and drinking water. This MIP sensor was constructed based on the multiple interactions formed between CLZ and two functional monomers, 2-vinylpyridine (2-VP) and MAA. In the same way, but without the use of nanomaterials, Toro et al., 2015 developed a selective sensor for hexazinone (HXZ) using computational simulations to evaluate the interaction of HXZ with functional monomers in the prepolymerisation mixture. MAA was selected as the most suitable monomer and this computational study allowed the production of a more effective MIP for HXZ with an excellent LOD of 2.6×10^{-3} nM.

3.2.3. Fungicides and biocides

Compared with insecticides and herbicides, the number of publications on the analysis of fungicides is much lower (see Table 2). An electrochemical MIP-sensor for the determination of dicloran in tap water and river water was developed by Khadem et al. (2016). In this work, the MIP produced by bulk polymerisation was deposited on top of a CNTs-modified CPE. The sensor showed very high recognition ability in comparison with a NIP electrode with a linear range between 1 and 1.0×10^3 nM and an LOD of 0.48 nM.

Besides this, a MIP sensor for tributyltin (TBT), a biocide used in antifoulant paints to prevent the growth of marine organisms on the hulls of large ships, was also described (Zamora-Gálvez et al., 2017). Fe₃O₄ MNPs were used to modify the MIP prepared by the sol-gel process using APTES as monomer. Using an SPCE as transducer and EIS as detection technique, a low LOD was achieved (5.4×10^{-3} nM).

3.3. Heavy metals

In developing countries, the increase of industrial activity and urbanisation has led to the proliferation of heavy metals in the soil and water sources (Ali et al., 2019a). The pollution of the environment with heavy metals has a particular impact on ecotoxicology because of their long persistence, non-degradability, bioaccumulation and biomagnification in the food chain, which causes serious harm not only to wildlife, but also to human health (Hong et al., 2020; Kumar et al., 2019). The MIP technology has attracted considerable attention to

assess and monitor the concentration of heavy metals (Liu et al., 2019; Yaroshenko et al., 2020). In the field of separation and sensing, several works involving the adsorption of heavy metals using MIPs can be found in the literature (Sharma and Kandasubramanian, 2020). However, as shown in Table 3, only a small number of publications concerning electrochemical MIP-based sensors to detect metal ions with applications in water and soil samples were reported. Electrochemical MIP sensors for the analysis of beryllium(II) (Be²⁺) (Li et al., 2015), cobalt(II) (Co²⁺) (Li et al., 2019), copper(II) (Cu²⁺) (Di Masi et al., 2020; Prasad and Fatma, 2016; Prasad and Singh, 2016) and zinc(II) (Zn²⁺) (Shirzadmehri et al., 2016) were developed following different strategies to facilitate metal ion imprinting and improve the selectivity of the sensor. The combination of MIP and ECL was reported for ultra-trace Be²⁺ detection in water samples (Li et al., 2015). The sensor was fabricated using Be²⁺ and 4-(2-pyridylazo)-resorcinol (PAR), a metallochromic indicator, to form a complex which was copolymerised with o-PD. CV characterisation of the prepared MIP sensor revealed that the imprinted cavities could only recognise the Be²⁺-PAR complex and not the metal ion and the ligand, showing an efficient imprinting process. ECL was indirectly measured by the luminol-H₂O₂ interaction and a low LOD (0.024 nM) was achieved. The same strategy for the detection of nanomolar levels of Co²⁺ was used by Li et al. (2019c). In their work, Co²⁺ and bovine serum albumin (BSA) were introduced as the template molecules and an ECL sensor was designed. Another attractive and different concept, based on biosorption, was described to detect Cu²⁺ in soil and water samples (Prasad and Fatma, 2016; Prasad and Singh, 2016). As certain algae show enormous potential for biosorption of heavy metals, Prasad and Fatma (2016) used a blue green-algae (*Aulospirra* sp.) modified PGE for the development of one MoNomer Ion Imprinted Polymer (OMNiMIP) (Fig. 5A). In this procedure, the functionalised algae were simultaneously used as crosslinker and functional monomer. The use of carboxylic groups in the OMNiMIP sensor facilitated the metal ion imprinting and a highly sensitive method with precise results was obtained. The absence of an extra crosslinker allowed the LOD to be several times lower than the one obtained by Prasad and Singh (2016). In this work, the same algae were employed as a natural ligand to form a “complex-metal” with N-methacryloylglutamic acid (NMGA) as the monomer for the analysis of Cu²⁺. The algae were considered as cheap substitutes of MWCNTs to impart electroconductivity to the film. Using differential pulse anodic stripping voltammetry (DPASV), an extremely sensitive technique used for the detection of heavy metal ions, Prasad and Singh (2016) developed a OMNiMIP without the need to select a crosslinker and, consequently,

Table 3
Electrochemical MIP sensors constructed on different sensing platforms for detection of heavy metals in water samples.

Target	Functional monomer	Polymerisation method/Transducer	Electrochemical techniques	Samples	Linear Range (nM)	LOD (nM)	Reference
Beryllium(II) (Be ²⁺)	o-PD	Electropolymerisation, SPAuE, Luminol-H ₂ O ₂	ECL	Rain, bottled and well water	0.07–8.0	0.024 ^a	Li et al. (2015)
Cobalt(II) (Co ²⁺)	o-AP	Electropolymerisation, Au electrode, BSA, MWCNTs, Cu, C-Dots	ECL	Surface water, industrial sewage and agricultural soil	1–1.0 × 10 ²	0.31 ^a	Li et al. (2019c)
Copper(II) (Cu ²⁺)	Acryloylated-algae NMGA	Bulk polymerisation, PGE	DPASV	Lake water and soil	0.13–1.2 × 10 ²	0.028 ^b	Prasad and Fatma (2016)
		Bulk polymerisation, PGE	DPASV	Lake water and soil	0.16–98	0.060 ^b	
	o-PD	Electropolymerisation, SPPE	DPV	Drinking water	0.95–2.4 × 10 ³	2.7 ^c	Di Masi et al. (2020)
Zinc(II) (Zn ²⁺)	MAA	Bulk polymerisation, CPE, GO, AgNPs, [BMP]Tf2N (IL)	Potentiometry	River water, industrial wastewater	3.9–1.0 × 10 ¹⁰	0.030 ^d	Shirzadmehri et al. (2016)

AgNPs: silver nanoparticles; [BMP]Tf2N (IL): ionic liquid 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide; BSA: bovine serum albumin; C-Dots: carbon dots; NMGA: N-methacryloylglutamic acid; o-AP: o-aminophenol; SPPE: screen printed platinum electrode.

^a LOD calculated as 3 times the standard deviation of the intercept divided by the slope.

^b LOD calculated as 3 times the standard deviation of the blank divided by the slope.

^c LOD calculated as 3 times the standard error from the weighted regression plot divided by the slope.

^d LOD formula not specified.

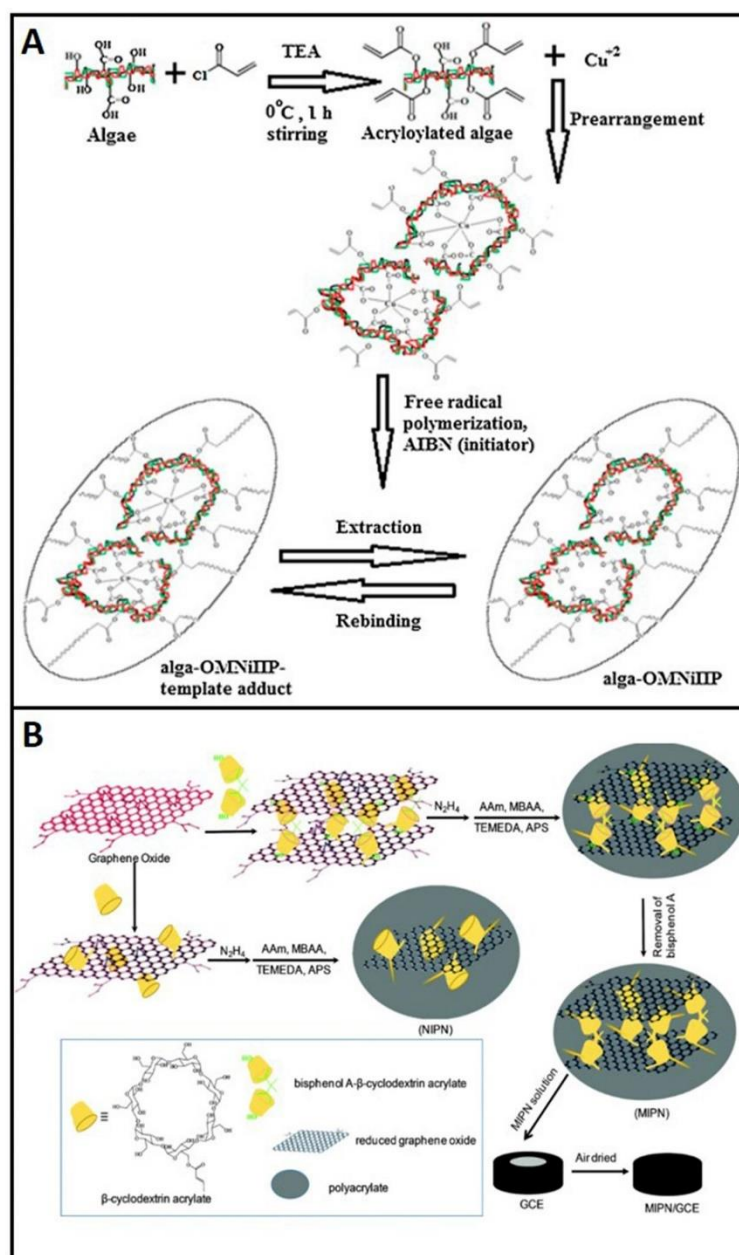


Fig. 5. A) Illustration of the preparation of an alga-OMNiMIP for electrochemical detection of Cu^{2+} . Reproduced from [Prasad and Fatma \(2016\)](#) with permission from Elsevier. B) Schematic illustration of the synthesis procedure of a MIP sensor for BPA analysis. Reproduced from [Ali et al. \(2019b\)](#) with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

without optimisation of the monomer-crosslinker ratio

3.4. Other contaminants

Besides pharmaceuticals, pesticides and heavy metals, the environment is also charged with other chemicals that may harm human health.

Many products of daily use, such as industrial explosives, sub-products of industrial syntheses, ingredients of cosmetics, brominated flame retardants or even amino acids have not surprisingly been detected in environmental matrices. As can be observed in [Table 4](#), a substantial amount of work has been done to develop novel sensors to determine the presence of these unwanted compounds in different environmental

Table 4
Electrochemical MIP sensors constructed on different sensing platforms for the detection of various contaminants in water samples.

Target	Functional monomer	Polymerisation method/Transducer	Electrochemical techniques	Supplies	Linear range (nM)	LOD (nM)	Reference
2,4,6-Trinitrotoluene	MAA	Bulk polymerisation, CPE	SWV	Tap water, ground water and soil	$5-1.0 \times 10^3$	1.5^a	Alizadeh et al. (2010)
Bisphenol A	MAA	Bulk polymerisation, CPE, Fe ₃ O ₄ MNPs	SWV	Tap and sea water	$1.0-1.3 \times 10^1$	0.5^a	Alizadeh (2014)
	MMA	Miniemulsion polymerisation, CPE, Fe ₃ O ₄ MNPs, CTAB	EIS	Drinking bottle and lake water	$6.0 \times 10^2-1.0 \times 10^5$	1.0×10^{2b}	Zhu et al. (2014)
	4-VP AA and MBAA Py	Bulk polymerisation, CPE, MWNTs Bulk polymerisation, GCE, β -CD, GO Electro polymerisation, GCE, QDs	DPV DPV DPV	River, tap and pure water Lake, tap and drinking water Tap and sea water	$80-1.0 \times 10^5$ $20-1.0 \times 10^3$ $1.0 \times 10^2-5.0 \times 10^4$	22^b 8^c 40^d	Chen et al. (2014b) Ali et al. (2019b) Tan et al. (2016)
Tetrabromobisphenol A	Py	Electro polymerisation, LSG electrode Sol gel method, GCE, GO	DPV	Mineral and tap water	$50-2.0 \times 10^4$	8^b	Beduk et al. (2020)
	APTES	Sol-gel method, GCE, GO	DPV	Mineral water	$6-1.0 \times 10^2$	3^b	Dadkhah et al. (2016)
	APTES APTES	Sol-gel method, CPE, SiO ₂ NPs Sol-gel method, GCE, Fe ₃ O ₄ -NCs	CV ECL	River water Seawater	$2.0 \times 10^2-2.0 \times 10^4$ $1.0 \times 10^2-5.0 \times 10^5$ $8.8 \times 10^{-3}-2.2 \times 10^4$	32^c 1.8×10^{-3a} 0.13^a	Wang et al. (2011) Zhang et al. (2020) Chen et al. (2014a)
Tetrabromobisphenol S	Py	Electro polymerisation, GCE, GO, NINPs	DPV	Tap water, rain and lake water	$0.5-1.0 \times 10^4$	0.13^a	Chen et al. (2014a)
	APTES	Sol-gel method, CPE, Fe ₃ O ₄ MNPs	DPV	Tap water, rain and pool water	$5.0-2.0 \times 10^3$	0.77^b	Zhou et al. (2016)
	MAA and PATP	Bulk polymerisation, CPE, AUNPs	DPV	Tap, lake and drinking water	0.1-10	0.029^b	Sarpog et al. (2020)
Sodium Lauryl sulfate	2-ATP	Electro polymerisation, SP/AuE	DVP	Wastewater and river water	0.1-3.5	6.3×10^{-4b}	Motia et al. (2018)
	o-PD	Electro polymerisation, GCE	CV	Tap and lake water	$10-1.2 \times 10^3$	4^d	Yu et al. (2019)
	Py	Electro polymerisation, GCE, GO	DPV	Rain and lake water	0.45-45	0.016^a	Chen et al. (2013)
Para-nitrophenol	MAA	Bulk polymerisation, CPE	DPV	River and tap water	$8-5.0 \times 10^3$	3^c	Alizadeh et al. (2009)
N-Nitrodimethylamine	Py	Electro polymerisation, GCE, SWCNTs	EIS	Bottled drinking water and tap water	$1.4 \times 10^2-3.1 \times 10^3$	11.5^d	Ceto et al. (2016)
	MAA	Bulk polymerisation, CPE	DPV	Tap water	$20-1.8 \times 10^2$	9.6^b	Aswini et al. (2014)
	AA	Bulk polymerisation, SP/AuE, PVC-COOH	DPV	Wastewater and mineral water	$3.5 \times 10^{-4}-3.5 \times 10^2$	7.9×10^{-4d}	Motia et al. (2019)
Methyl green dye	AA	Bulk polymerisation, CPE, Fe ₃ O ₄	SWAdASV	River water and industrial wastewater	$99-1.8 \times 10^3$	10^c	Khan et al. (2019)
Diphenylamine	(VC4min)[PP6] (IL)	Bulk polymerisation, GCE, RGO, Fe ₃ O ₄	DPV	Lake water	$1.0 \times 10^2-1.8 \times 10^3$	50^a	Liu et al. (2018)

2-ATP: 2-aminothiophenol; 4-VP: 4-vinylpyridine; AA: acrylamide; CTAB: Cetyltrimethylammonium bromide; MBAA: N,N'-methylenebis-acrylamide; NCs: nanocrystals; NINPs: nickel nanoparticles; LSG: laser scribed graphene; PVC-COOH: carboxylic poly(vinyl chloride); RGO: reduced Graphene oxide; SiO₂ NPs: silica nanoparticles; SWAdASV: square-wave adsorptive stripping; SWCNTs: single walled carbon nanotubes; (VC4min)[PP6] (IL): ionic liquid 1-vinyl-3-butylimidazolium hexafluorophosphate.

2D hBN: two dimensional hexagonal boron nitride; 2-VP: 2-vinylpyridine; APTES: (3-Aminopropyl)triethoxysilane; Ag-N@ZnO: silver and nitrogen co doped zinc oxide; C3N4 NTs: carbon nitride nanotubes; CFP: carbon fiber paper; Ch: chitosan; CHAC: activated carbon prepared from coconut husk; CMPE: carbon paste microelectrode; CNTs: carbon nanotubes; CPE: carbon paste electrode; CV: cyclic voltammetry; DA: dopamine; DPASV: differential pulse anodic stripping voltammetry; EBB: eriochrome blue-black B; ECL: electrochemiluminescence; EDOT: 3,4-ethylenedioxythiophene; Fe@AUNPs: core-shell type nanoparticles; GQDs: graphene quantum dots; LSV: linear stripping sweep voltammetry; MAC: N-methacryloyl-L-cysteine; N-Gs: nitrogen doped graphene sheets; Ph: phenol; Pt-Uio-66: Zr-based metal-organic framework catalyst; QDs: quantum dots; RC: resorcinol; ZnO NPs: zinc oxide nanoparticles.

^a LOD calculated as $S/N = 3$.

^b LOD calculated as 3 times the standard deviation of the blank divided by the slope.

^c LOD formula not specified.

^d LOD calculated as 3 times the standard deviation of the blank.

water samples. In this section, emphasis will only be given to new design strategies of MIPs.

Since 2011, bisphenol A (BPA) has attracted widespread attention as contaminant. It is mainly used to produce epoxy resins and polycarbonate plastics and is found in bottles, food containers and toys. BPA is considered an endocrine disruptor and human exposure leads to a variety of health issues (Ali et al., 2019b). Various electrochemical strategies have been used for the selective determination of BPA in different types of waters (Ali et al., 2019b; Chen et al., 2014b; Dadkhah et al., 2016; Tan et al., 2016; Wang et al., 2011; Zhu et al., 2014) (see Table 4). There is no consensus on the technique used for its molecular imprinting, but the presence of nanomaterials was essential for the adequate performance of the sensors. Recently, Ali et al. (2019b) reported a very robust MIP based on a nanocomposite of polyacrylate, reduced GO (rGO) and β -cyclodextrin that were covalently linked forming a 3D network (Fig. 5B). β -cyclodextrin is known to form an inclusion complex with BPA by means of host-guest complexation, while polyacrylate allows to form porous 3D networks. The first step of the synthesis consisted of the reaction between GO and β -cyclodextrin in the presence of BPA; the hydroxyl groups of β -cyclodextrin strongly react with the epoxide groups of GO and a stable interaction is obtained. Then, radical polymerisation was performed using two functional monomers, acrylamide (AA) and N,N' -methylenebis-acrylamide (MBAA). The electrochemical detection of BPA was performed using GCE as transducer, reaching an interesting LOD (8 nM). In this work the combination of rGO and β -cyclodextrin in molecular imprinting was explored for the first time, providing promising insights for the design of more selective MIPs.

The same LOD was observed with a low-cost imprinted sensor based on laser scribed graphene (LSG) technology (Beduk et al., 2020). This device was used as transducer to detect BPA after electropolymerisation of Py. Dadkhah et al. (2016) and Wang et al. (2011) developed strategies to prepare MIPs on the surface of GO and hexagonally structured mesoporous silica, respectively, to enhance the electrochemical signal. In both works, amine functionalisation of GO and SiO_2 NPs was achieved by a simple procedure using APTES to improve the recognition ability of the sensors. After functionalisation, the targets were immobilised on its surface and not only many homogenous imprinting sites were formed, but also an easy access of the analyte molecules to the imprinting sites was reported. At the same time, the best LOD found for BPA was 3 nM using GO in the modification of the electrochemical MIP sensor. Recently, ECL again showed its potential to achieve a higher sensitivity (Zhang et al., 2020). These authors developed a MIP sensor based on a GCE modified with Fe_3O_4 nanocrystals (Fe_3O_4 -NCs), which increased the ECL response signal of luminol. The proposed MIP sensor displayed an excellent analytical performance and a low LOD (1.8×10^{-3} nM) was observed.

Another compound that is widely used in epoxy and polycarbonate resins is tetrabromobisphenol A (TBBPA), which is the most common brominated flame retardant on the market (Zhou et al., 2016). TBBPA has also been linked with endocrine disruption. For its electrochemical detection, Chen et al. (2014a) presented a MIP based on nickel nanoparticle-modified GO and showed that the combination of more than one nanostructured material increased the effective surface that can be an attractive route for the preparation of MIP-based sensors with adequate sensitivities.

Incorporation of ionic liquids (ILs) in the MIP framework has also been the focus of many studies. By exploring their good electrocatalytic activity, adsorption capacity and multiple interactions with targets, there are some works adopting polymerisable ILs as functional monomers and crosslinkers to prepare MIPs (Ding et al., 2020). Liu et al. (2018) introduced a new IL composite using IL 1-vinyl-3-butylimidazolium hexafluorophosphate ([VC4mim][PF6]) as functional monomer, IL 1,4-butanediyl-3,3'-bis-1-vinylimidazolium dihexafluorophosphate ([V2C4(mim)2][PF6]2) as crosslinker and GO and Fe_3O_4 as support, to detect diphenylamine (DPA) in water samples. In their work the authors showed that the proposed MIP based on such IL as crosslinker had a

higher electrochemical response than that of the traditional ethylene glycol dimethacrylate. Although, ILs have been considered alternative green solvents, it is important to note that ILs are quite expensive and their recycling is difficult.

Another strategy in surface imprinting was shown by Motia et al. (2019) through the functionalisation of an SPAuE with a carboxylic polyvinyl chloride (PVC-COOH) layer for the detection of triclosan (TCS), which is an antibacterial agent incorporated in many products of daily use. After linking TCS with PVC, strong hydrogen bonds between the polar groups of TCS and -COOH groups of the PVC were established.

4. Conclusions and perspectives

Environmental water quality monitoring is essential to ensure the safety of the aquatic environment and, consequently, human and animal health. Moreover, the analysis of contaminants in the environment is essential to know their pathways, fate and effects, but also to determine the WWTP's removal efficiency.

In this review, the considered studies demonstrated a significant increase in the development of MIP-based electrochemical sensors to detect contaminants in water samples. MIPs are polyvalent receptors, which are easily synthesized, modified and manipulated and, more importantly, they are compatible with water. The MIP's sensitivity and affinity are usually improved by their combination with nanomaterials. This has stimulated researchers to design and create new and innovate MIP sensors with the ability to recognise the target molecules at trace levels. However, although MIPs have proven their potential as recognition elements, the need of extremely low LODs remains an open challenge. This is the reason why many MIP sensors for determination of pharmaceuticals are not applied to water analysis but to serum and/or urine (for clinical control) or pharmaceutical formulations (for quality control).

Regarding the imprinting process, there is no specific approach for a particular class of molecules. So, the synthesis process and functional monomer that provide the best MIP for the target molecule have to be obtained by experimental studies. In this context, computational studies, reported only in few publications, are increasingly used since they have proven to be valuable for selecting suitable functional monomers. Besides the fact that computational modelling allows a better understanding of the monomer-template interactions, it provides important guidelines and, therefore, leads to an overall more environmentally friendly process for MIP construction. Accordingly, more efforts should be made to exploit theoretical approaches to pre-screen the preparation conditions, since good selectivities and LODs are achieved.

Regarding the transducers, carbon electrodes are undoubtedly the most widely reported sensing platform for the determination of contaminants. Regarding the detection strategy, the highest sensitivity is usually attained by indirect electrochemical sensing, although this strategy can have some drawbacks, such as false results due to the presence of masked interferents.

Although MIPs are highly selective, this can also be a disadvantage when the determination of several contaminants is required. Thus, the development of MIPs capable to recognise more than one contaminant (for example, a family of contaminants with similar molecular structures) could be useful for some applications. Another approach could be the development of multiplex MIP-based sensors, although nowadays they are scarce for environmental applications. Moreover, portable electrochemical sensors show another difficulty for their application in environmental analysis: analytes have to be extracted from solid samples (such as soils) to allow analysis. Liquid samples, mainly water, are easier to analyse (since they are aqueous); however, sometimes they present a complex matrix that could interfere in the analysis.

Therefore, continuous studies within multidisciplinary teams must be performed to develop highly sensitive and accurate methods, aiming to implement MIPs in the analysis of contaminants in environmental waters. The construction of portable MIP sensors, especially by using

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screen printed electrodes, could be very useful in field applications. However, the commercialisation of these sensors remains a challenge, since, as for other kind of sensors (biological sensors and sensors for food applications), the knowledge transfer from laboratories to the market is difficult.

Declaration of competing interest

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

Acknowledgements

The authors are grateful for the financial support from the Fundação para a Ciência e a Tecnologia (FCT)/the Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) through national funds (Portugal) (UIDB/50006/2020 and UIDP/50006/2020). The authors would also like to thank to the EU and FCT/UEFISCDI/FORMAS for funding in the frame of the collaborative international consortium REWATER, financed under the ERA-NET Cofund WaterWorks 2015 Call. This ERA-NET is an integral part of the 2016 Joint Activities developed by the Water Challenges for a Changing World Joint Programme Initiative (Water JPI). This work was also supported by the project Farmasense (39957), funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020) and the Fundo Europeu de Desenvolvimento Regional (FEDER). Isabel Seguro received her research Grant through this project. E. Costa-Rama thanks the Government of Principado de Asturias and Marie Curie-Cofund Actions for the post-doctoral grant “Clarín-Cofund” (ACA17-20). Patrícia Rebelo is grateful to FCT for her PhD grant (SFRH/BD/132384/2017). João Pacheco is grateful to FCT for his postdoc grant (SFRH/BPD/101419/2014), financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu and MCTES.

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Chapter 3

RATIONAL DEVELOPMENT OF ELECTROCHEMICAL MIPs SENSORS FOR PHARMACEUTICALS DETECTION

3.1 Azithromycin electrochemical detection using a molecularly imprinted polymer prepared on a disposable screen-printed electrode

3.2 Rational development of molecular imprinted carbon paste electrode for furazolidone detection: theoretical and experimental approach

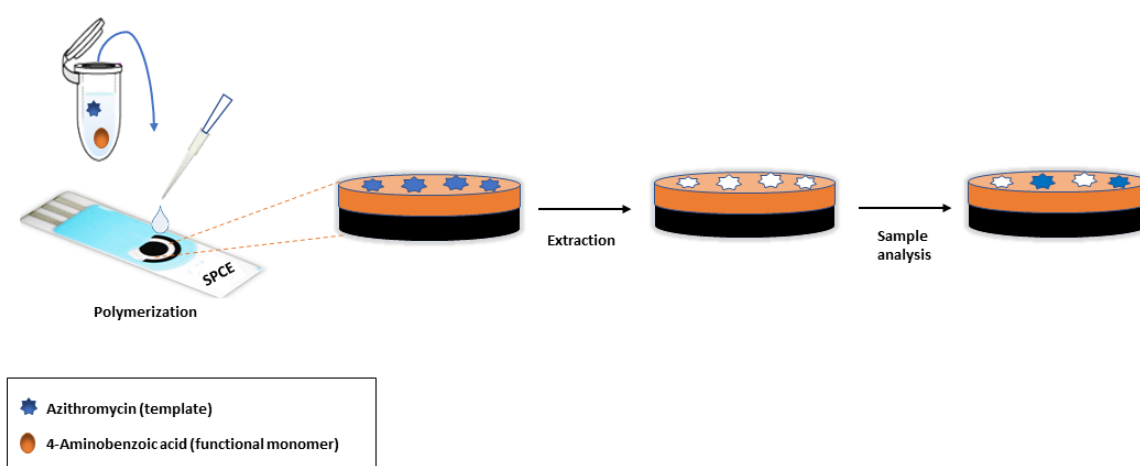
3.3 Development of a molecular imprinted electrochemiluminescence sensor for amitriptyline detection: from MD simulations to experimental implementation

3.4 A simple electrochemical detection of atorvastatin based on disposable screen-printed carbon electrodes modified by molecularly imprinted polymer: experiment and simulation

3.5 Computational modelling and sustainable synthesis of a highly selective electrochemical MIP-based sensor for citalopram detection

3.1 Azithromycin electrochemical detection using a molecularly imprinted polymer prepared on a disposable screen-printed electrode

Analytical Methods 12 (11) (2020) 1486-1494



Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review, investigation, methodology and writing of the original draft.



Cite this: DOI: 10.1039/c9ay02566a

Azithromycin electrochemical detection using a molecularly imprinted polymer prepared on a disposable screen-printed electrode

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Azithromycin (AZY) is among the antibiotics with the highest concentrations in aqueous matrices. Conventional wastewater treatment plants (WWTPs) do not fully remove this compound and it has been found in the environment. The health effects of AZY on humans and the environment are not yet understood. Therefore, the development of sensitive and selective analytical methods for AZY determination is important. In this work, an electrochemical molecularly imprinted polymer (MIP) sensor was developed for the quantification of AZY. The selection of the functional monomer was performed by molecular modelling using quantum mechanics calculations. The MIP was electropolymerized by cyclic voltammetry (CV) on a screen-printed carbon electrode (SPCE) using a solution containing 4-aminobenzoic acid (4-ABA) in the presence of AZY as a template molecule. The characterisation of the sensor was carried out using scanning electron microscopy (SEM), CV and electrochemical impedance spectroscopy (EIS). The analysis of AZY was performed by differential pulse voltammetry (DPV) in the linear range between 0.5 and 10.0 μM , with a limit of detection (LOD) of 0.08 μM ($S/N = 3$) and a limit of quantification (LOQ) of 0.3 μM ($S/N = 10$). The MIP sensor was found to have selectivity to recognise AZY molecules and was successfully applied in the analysis of tap water and water samples collected upstream of a WWTP output in the Ave River. The proposed sensor presents a simple, selective and environmentally friendly strategy for AZY determination in environmental water.

Received 28th November 2019
Accepted 22nd February 2020

DOI: 10.1039/c9ay02566a

rsc.li/methods

1. Introduction

Pharmaceutical compounds have become a great global concern in recent years due to their unintentional presence in aquatic environments.^{1–4} With the continuous growth of the world population, they are extensively and increasingly used, not only to prevent or treat human and animal diseases, but also as growth promoters in the livestock and aquaculture industries.^{3,5,6} These compounds have emerged as important contaminants, because they have been accumulating in the environment through different routes.^{3,7,8}

Antibiotics are one of the most important groups of pharmaceuticals in our daily lives.^{7,9,10} Since most antibiotics are water-soluble and only a part of the administered dose is metabolized, approximately 90% of a dose can be excreted in urine and up to 75% in animal excrement.^{7,11} Then, they are discharged into wastewater collection systems as unchanged compounds, together with a mixture of their metabolites.¹²

Drug manufacturers and hospitals are the major sources of contaminated wastewaters.⁷ Consequently, most antibiotic residues have already been detected in different water sources, including surface water, groundwater and drinking water¹ at concentrations ranging from ng L^{-1} to mg L^{-1} .^{5,6,13,14} Even though concentrations are generally considered very low, the long-term persistence of antibiotics raises concerns about the proliferation of antibiotic resistance.^{14–19}

Azithromycin (AZY) is an azalide belonging to a class of 15-membered macrolide antibiotics, and is used to treat many different bacterial infections. These include respiratory, ear and skin infections. This antibiotic is especially effective for treating chronic inflammatory disorders.²⁰ After being consumed, a major part of this drug is mainly eliminated unchanged *via* the biliary tract and, to a lesser extent, in urine.²¹ In addition, its recalcitrance was demonstrated during wastewater treatment with an average removal of 14%.²² AZY is among the antibiotics with the highest concentrations in aqueous matrices,^{23,24} which explains its inclusion in the EU Watch List (Decision 2018/840/EU) of substances that could pose a significant risk to aquatic environments.^{14,22,25} Therefore, it is very important to develop sensitive and selective analytical methods for accurately tracing this contaminant in the environment. Several methods have been developed for this purpose. These include bioassays,

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Analytical Methods

spectrophotometry and high-performance liquid chromatography (HPLC) with ultraviolet detection or fluorescence detection. However, most of these methods present severe limitations in relation to sensitivity and cost-effectiveness. Additionally, they require a complicated extraction or derivatization procedure and a long analysis time.

So, electrochemical sensors have drawn increasing attention, because of their high sensitivity, fast response, ease of preparation and real-time detection.²⁶ Additionally, they represent a much lower cost and improved field portability.²⁷ In the last few years, the combination of molecularly imprinted polymers (MIPs) with electrochemical sensors has attracted considerable interest. MIPs offer some clear advantages including better stability with a high surface area over a wide range of experimental conditions and solvents, becoming powerful alternatives to biorecognition elements.²⁶ Thus, novel and innovative reports on MIPs have emerged for detection of a wide variety of significant analytes such as drugs,^{28–30} biomarkers,³¹ neurotransmitters³² and pesticides.^{33,34} Molecular imprinting is able to create highly specific and long-life time recognition cavities on the sensor surface, through a process where selected functional monomers are polymerized around a target analyte (template).³⁵ A stable template-monomer complex is key for strong molecule selectivity and binding properties of a MIP.³⁶ Thus, the choice of functional monomer is crucial for the success of molecular recognition.^{37–39} Functional monomer screening using computational models has become very important for a rational MIP design protocol.^{40,41} Computational modelling allows understanding of the impact of polymerization on the structure of monomer-template complexes and selection of the best monomer for a polymer preparation.⁴⁰ Additionally, this approach is much more sustainable, as it minimises the laboratory tasks and the amount of organic solvents used.⁴²

Nowadays, efforts are being made to find environmentally friendly processes in line with green chemistry concepts. These include the reduction or elimination of toxic or hazardous reagents, solvents and waste products.⁴³ In this work, a simple and selective MIP sensor, for AZY determination, was developed by electropolymerization of 4-aminobenzoic acid (4-ABA) in aqueous solutions directly on the surface of a screen-printed carbon electrode (SPCE). An SPCE has advantages over traditional electrodes due to the possibility of use in miniaturized systems, where low volume samples are required. The monomer was selected by computational studies, where association energies between multiple monomers and AZY were calculated in order to select a more appropriate functional monomer for building a sensor with higher affinity to AZY. The developed sensor is suitable for application in commercial analytical solutions and adheres to the principles of sustainable chemistry.^{43,44}

2. Experimental

2.1 Reagents

AZY was obtained from Sigma-Aldrich. A stock solution of AZY was prepared by weighing an appropriate amount of the

antibiotic and dissolving it in 2 : 1 methanol–water (MeOH–H₂O) and stored at 4 °C. Working solutions were prepared daily by adequate dilution of the stock solution in a phosphate buffer (0.1 M, pH = 8) and kept refrigerated. For interference studies, clarithromycin (CLY), erythromycin (ERY), streptomycin sulphate (STRE) and tetracycline (TETRA), obtained from Sigma-Aldrich, were used. 4-ABA and hydrochloric acid (HCl) were also purchased from Sigma-Aldrich and MeOH (≥99.9%) from Merck.

All chemicals were used as supplied without any further purification. Ultra-pure water (resistivity = 18.2 MΩ cm) obtained from a Millipore (Simplicity 185) water purification system was utilized in all experiments. Phosphate buffer solution (0.1 M, pH = 7) was prepared with KH₂PO₄ and K₂HPO₄ (Riedel-de-Haën) and used as the supporting electrolyte.

The water samples were collected from a tap and upstream of a WWTP output in the Ave River.

2.2 Apparatus

All voltammetric measurements were performed at room temperature using a Metrohm Autolab PGSTAT 204 potentiostat/galvanostat controlled by NOVA 1.10 software. Electrochemical impedance spectroscopy (EIS) was performed using a Metrohm Autolab PGSTAT 128 N potentiostat/galvanostat controlled by NOVA 1.6 software. The screen-printed carbon electrodes used in this study were purchased from Metrohm AG (SPCEs, DRP-110). Each of them consisted of a 4 mm carbon working electrode, a graphite auxiliary electrode and a silver pseudo-reference electrode. The specific connector (DRP-CAC) to interface the SPCE and the potentiostat/galvanostat was supplied by DropSens. The pH measurements were carried out using a Crison pH meter (model microPH 2002) with a combined pH glass electrode. SEM studies were carried out at CEMUP (Centro de Materiais da Universidade do Porto). SEM images were obtained with a Quanta 400 FEG scanning electron microscope (SEM, FEI, Hillsboro, OR), operated in high vacuum/secondary electron imaging mode using an accelerating voltage of 25 kV and working distances of around 6.6 mm.

2.3 Molecular modelling

Five monomers, 4-ABA, pyrrole, aniline, thiophene and phenol, were studied as potential functional monomers for AZY-MIP film formation. The molecular fragments, each of them including a monomer and the template (AZY), were modelled using VMD.⁴⁵ The monomers were then docked into the template to generate the respective complexes with AZY, using the program AutoDock⁴⁶ within the VMD plugin vsLab.⁴⁷ The best conformation was used for further analysis and characterization using quantum mechanics calculations. Their structures were initially pre-optimised using the Gaussian 09 program⁴⁸ at a PM6 or a PDDG semi-empirical level. The geometries obtained were further fully optimised using the same program at an appropriate M06-2X/6-31+G* density functional theory (DFT) level. This functional is appropriate for comparing the relative stability of the complexes formed under study, once it can describe properly the dispersive interactions

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that are dominant in these molecular systems. For each optimised geometry, harmonic frequencies were also calculated at the same theoretical level to confirm that it corresponds to a genuine minimum by checking that they were positive.

2.4 Sensor fabrication

Fig. 1 illustrates the fabrication steps of the AZY sensor. Under the optimum conditions, the electropolymerization of the MIP-sensor was performed on the SPCE surface using cyclic voltammetry (CV) in the potential range from -0.2 to 1.4 V, for 10 consecutive cycles, at a scan rate of 100 mV. A drop of 40 μ L of a solution containing 0.05 mM AZY antibiotic and 5 mM 4-ABA, as a functional monomer in a phosphate buffer ($\text{pH} = 7$), was used as the polymerization medium. The electrodes were then rinsed with ultra-pure water and a phosphate buffer solution of $\text{pH} 10$ was dropped on the electropolymerized film for 10 min, to remove the AZY entrapped in the polymer matrix. A non-imprinted polymer (NIP) electrode was prepared using the same experimental conditions, but without adding AZY into the polymerization solution, to check the reliability of measurements.

3. Results and discussion

3.1 Selection of the functional monomer

The noncovalent imprinting approach is widely used in MIP preparation, due to its simplicity. A stronger interaction between the monomer and template before polymerization may lead to a better imprinting effect. So, a successful molecular imprinting process is strongly dependent on the selection of a suitable monomer. For this purpose, computational modelling was used here as a valuable pre-screening rational design strategy. This enabled us to select the monomer with the best chemical complementarity with the template, using theoretical calculations.

The five monomers (4-ABA, pyrrole, aniline, thiophene and phenol), and the respective complexes with AZY, were then studied using the molecular modelling procedure described in Section 2.3. The lowest energy conformation was selected as representative of each complex. The corresponding association energies (ΔE) can be calculated as follows:

$$\Delta E = E(\text{complex}) - E(\text{template}) - E(\text{monomer}),$$

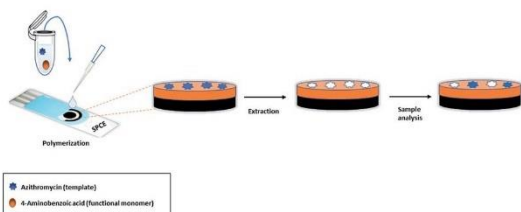


Fig. 1 Schematic illustration of the preparation of the MIP/SPCE sensor.

Table 1 Association energies (ΔE) for the AZY–monomer complexes, as well as the number (n) of hydrogen bonds responsible for their stabilization^a

Complex	$\Delta E/\text{kJ mol}^{-1}$	n
AZY–4-ABA	-71.55	2
AZY–phenol	-69.41	1
AZY–pyrrole	-68.93	1
AZY–aniline	-45.08	1
AZY–thiophene	-31.96	0

^a These results were obtained, using Gaussian 09, at an M06-2X/6-31+G* DFT level.

where $E(\text{complex})$ is the energy of the complex, $E(\text{template})$ the energy of AZY and $E(\text{monomer})$ the energy of the monomer.

Table 1 presents the association energies, for the AZY–monomer complexes, as well as the lengths of the hydrogen bonds responsible for their stabilisation. These results enabled us to order the monomers in the ascending order of the respective affinity for the template, i.e.: $\Delta E(\text{thiophene}) < \Delta E(\text{aniline}) < \Delta E(\text{pyrrole}) < \Delta E(\text{phenol}) < \Delta E(4\text{-ABA})$. These results indicate that 4-ABA is expected to present the highest affinity for AZY ($\Delta E = -71.548$ kJ mol^{-1}).

The preferential stabilisation of the AZY–4-ABA complex can be associated with the strong hydrogen bonds established between the hydrogen of the hydroxyl group in 4-ABA and the oxygen of a hydroxyl group in AZY and between the hydrogen of the dialkylamine group in 4-ABA and the oxygen of another hydroxyl group in AZY (see Fig. 2).

According to the above-mentioned theoretical results, 4-ABA should present the highest selectivity for the AZY template. Therefore, only this monomer was used for molecular imprinting of AZY.

3.2 Molecular imprinting and electrochemical behaviour of AZY

The formation of the polymeric film was carried out by electropolymerization of a solution containing 0.05 mM AZY and

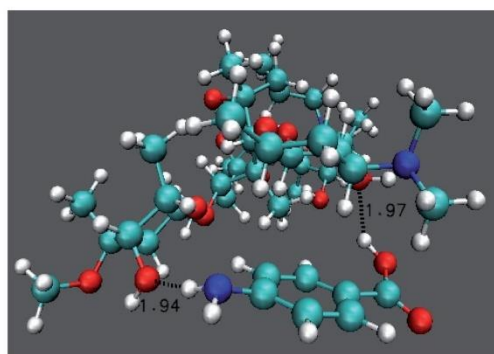


Fig. 2 Optimised structure for the AZY–4-ABA complex obtained, using Gaussian 09, at an M06-2X/6-31+G* DFT level.

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5 mM 4-ABA in phosphate buffer (pH = 7). 40 μL of this polymerization solution was placed on the surface of the SPCE and ten cyclic voltammetry scans were conducted between -0.2 and 1.4 V at a scan rate of 100 mV s^{-1} , forming a polymeric film with AZY entrapped molecules, as shown in Fig. 3A. The first scan showed the highest oxidation peak at about $+0.80$ V and a reduction peak at around -0.1 V.

During the second scan, another oxidation peak at around $+0.35$ V was observed. Those peaks indicated the successful formation of a polymeric film in the surface of the SPCE.

As the number of scans increased, a continuous growth of the polymeric chain was observed, with a constant decrease in the peak's current intensity. No significant differences were observed between the formation of MIP and NIP films.

After electropolymerization and before template removal, a DPV analysis was performed to demonstrate the incorporation of AZY. The results illustrated in Fig. 3B show a significant difference between the signals obtained with the MIP and NIP sensors. In the DPV analysis of the MIP, immediately after polymerization, an oxidation peak at $0.7\text{--}0.8$ V was registered while in the NIP no peak was observed. The peak in the MIP corresponds to the oxidation of entrapped AZY molecules, proving the successful incorporation into the polymer. After this step, the AZY molecules were removed from the MIP sensor using 0.1 M phosphate buffer at pH 10. This procedure ensured that highly specific recognition sites complementary in shape, size and functionality of AZY were produced. As expected, the peak attributed to the oxidation of AZY molecules disappeared. This confirmed an efficient extraction and the formation of binding cavities.

The rebinding of AZY molecules to both MIP/SPCE and NIP/SPCE was finally tested, using a 0.1 M phosphate buffer solution (pH = 7) after 10 min of incubation in 0.05 mM AZY. The results are shown in Fig. 3C. It can be observed that the oxidation peak current intensity obtained with the MIP was higher than the peak current for the NIP. This result indicates that the MIP-sensor has specific size and shape cavities, which allow more AZY molecules to bind at the surface. The peak observed after incubation in the NIP/SPCE indicates the ability of poly 4-ABA to non-specifically bind to AZY. So, there is a clear difference

between the NIP and MIP, demonstrating the effective preparation of a molecularly imprinted sensor.

3.3 SEM characterization of the electrodes

SEM analysis was performed to evaluate the morphology of the prepared sensor. Fig. 4A shows a rough surface characteristic of the bare SPCE. After polymerization of MIP/SPCE, there is formation of a very thin polymer layer, as can be observed in Fig. 4B by the slight thickening of the rough surface of the SPCE. After template extraction, no significant differences were observed (Fig. 4C). These images confirm that a thin polymer was formed under the used conditions and it was not removed during the template extraction.

3.4 CV and EIS characterization

A step by step characterization of the sensor construction was performed by CV and EIS, using a solution of 1 mM

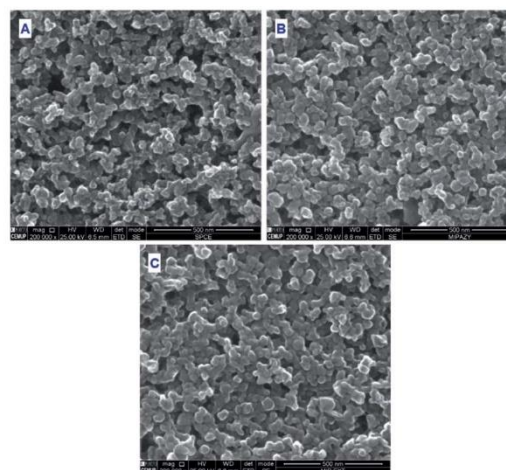


Fig. 4 SEM images of the (A) SPCE, (B) MIP/SPCE and (C) MIP/SPCE after template extraction.

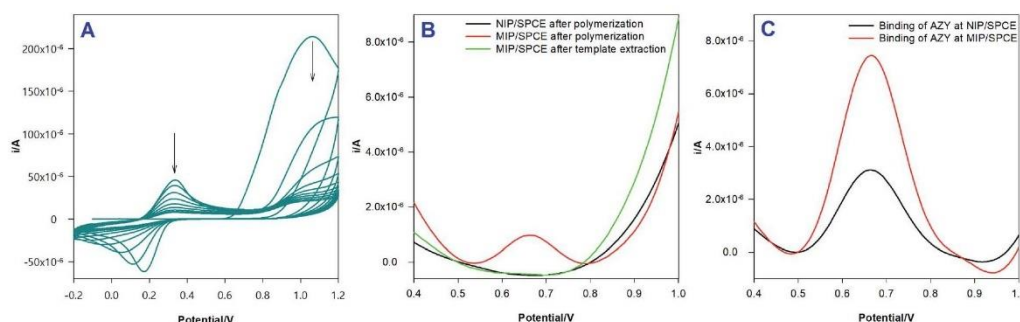


Fig. 3 (A) Electropolymerization of the MIP from a solution containing 10 mM 4-ABA and 0.5 mM AZY in 0.1 M phosphate buffer at pH 7; (B) DPV analysis of NIP and MIP sensors after polymerization; (C) DPV analysis of NIP and MIP sensors after rebinding to AZY.

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$[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M pH = 7 phosphate buffer solution (Fig. 5). As expected, two well-defined redox peaks were observed for the unmodified SPCE, as shown in Fig. 5A. After polymerization, both MIP and NIP sensors show a decrease in the current response, indicating a blockage of the electron transfer by the polymer, and thus confirming the success of formation of a polymer film in the surface of the electrode. The efficiency of AZY molecule removal was confirmed by the subsequent increase in the redox peaks. The formation of cavities by removal of AZY molecules allows more $[\text{Fe}(\text{CN})_6]^{3-/4-}$ molecules to reach the surface of the electrode, and thus the peak current increases. Finally, the rebinding of AZY molecules was confirmed by a decrease of the redox peaks. This can be explained by the fact that the AZY molecules occupy some of the cavities and block the diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

In EIS, the semicircle of the Nyquist diagram for higher frequencies corresponds to the electron transfer process, while the linear part of the lower frequency corresponds to diffusion. In Fig. 5B, the straight line for the SPCE corresponds to a diffusion limited process with fast electron transfer kinetics. After polymerization, there was an increase of the impedance of both the MIP and NIP. The resulting surface was highly resistant to electron transfer due to the formation of the polymer film. After AZY extraction, a decrease of the impedance was observed. This can be explained by the formation of cavities after AZY removal, improving the diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ through the polymer and facilitating the electron transfer process. Finally, after the rebinding of AZY a slight increase of the semicircle occurred. This indicated that AZY was successfully bound to the MIP and occupied some of the selective cavities.

3.5 Optimisation of the preparation conditions

Several important parameters for the performance of the MIP sensor were investigated and optimised by DPV. These include the monomer (4-ABA) and template (AZY) concentrations, the extraction and incubation conditions and the number of polymerization cycles.

Using different concentrations of the monomer (0.5, 1, 5, and 10 mM) in the polymerization solution, it was observed that the electrochemical performance of the prepared MIP is strongly dependent on this parameter. The sensor response increased up to a 4-ABA concentration of 5 mM and significantly decreased for higher concentrations (see Fig. 6A). This indicated that 5 mM is the optimal concentration for this monomer. The influence of template concentration was studied in the range from 0.01 to 5 mM, as shown in Fig. 6B. The maximum sensor response was found for an AZY concentration of 0.05 mM, which was chosen as the optimum template concentration.

A significant decrease in the peak current was registered for higher and lower concentrations. At higher concentrations, this tendency probably indicates the entrapping of too many template molecules in the polymeric film, making their extraction more difficult.

Electropolymerization is a very simple procedure for surface imprinting, where control over the polymer thickness can be achieved by adjusting the number of potential cycles. So, different numbers of CV cycles, ranging from 5 to 30, were evaluated. As shown in Fig. 6C, the peak current intensity of the MIP sensor is higher with 10 cycles and then a continuous decrease was observed. This decrease can be explained by an increase in the thickness of the polymeric film, which makes it difficult to extract AZY molecules imprinted close to the surface. For this reason, 10 cycles were used.

The extraction of template molecules entrapped in the polymeric matrix is an essential step to obtain a selective and efficient MIP.

In this work, several solvents were tested to remove AZY molecules, including 0.1 M phosphate buffer at pH 10, MeOH, 0.1 M HCl and a 0.1 M HCl/MeOH (50 : 50, v/v) mixture. The

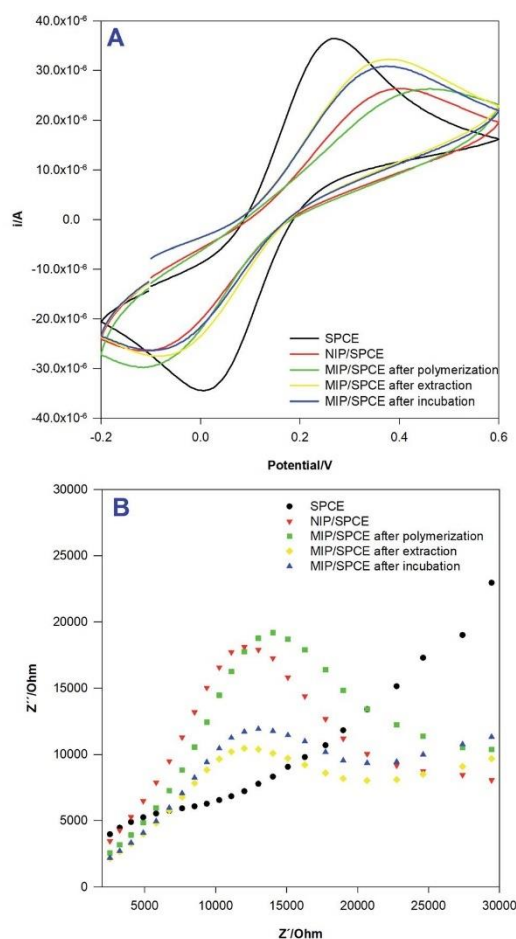


Fig. 5 Step by step construction characterization. (A) CV voltammograms and (B) EIS Nyquist diagrams in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M phosphate buffer at pH 7.

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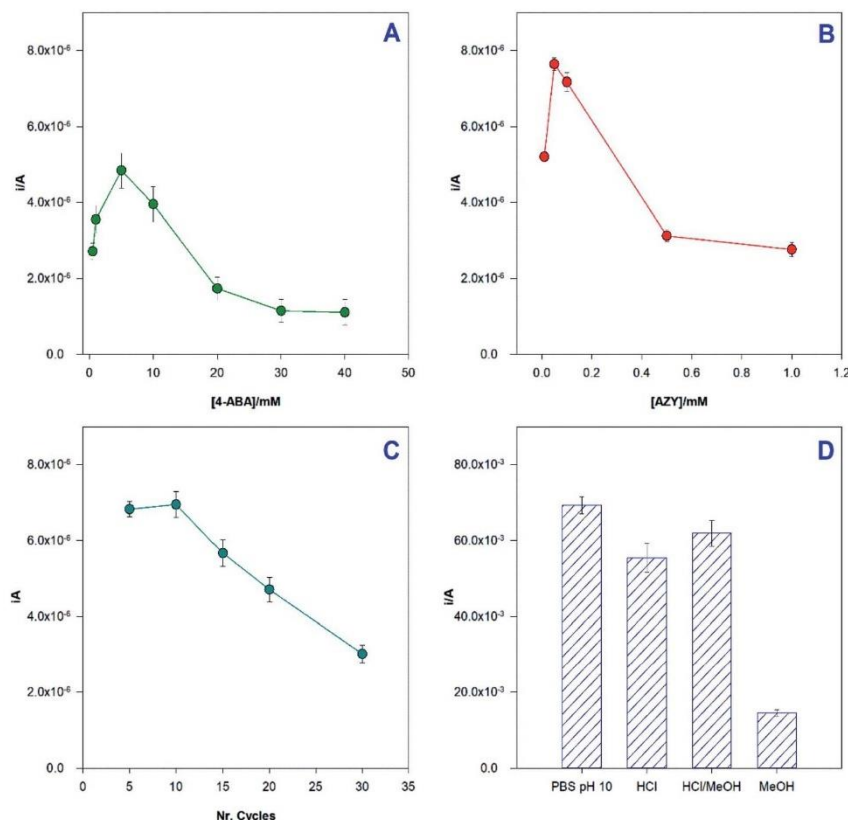


Fig. 6 Optimisation of the MIP preparation conditions: (A) variation of the peak current intensity with the concentration of monomer 4-ABA; (B) variation of the peak current intensity with the concentration of template AZY; (C) variation of the peak current intensity with the number of CV cycles and (D) variation of the peak current intensity with the extraction solvent.

removal of template molecules of binding cavities was considered complete when no oxidation peak appears in DPV analysis. After extraction with the tested solvents, the incubation and DPV analysis of AZY was performed. The results are shown in Fig. 6D; it is possible to conclude that a better sensor response was achieved with the 0.1 M phosphate buffer at pH 10.

On the other hand, the worst extraction was obtained with MeOH. It was also found that 10 min is the optimal extraction time. No significant differences were observed when longer extraction times were tested.

The influence of incubation conditions (solution pH and time) was also evaluated because it affects the adsorption of AZY molecules on the MIP sensor.

The effect of pH value of the incubated sample was investigated in the pH range from 2.0 to 10.0. It was observed that the peak current gradually increased as the pH increases; however, for a pH of 10.0, the NIP and MIP signals were similar, meaning that under this condition the contribution of non-specific binding is the most important. A pH of 8 was chosen for the rebinding of AZY molecules. Different incubation times (2.5, 5,

10, 20, and 30 min) were also studied. An increase in the sensor response was observed up to 15 min and then a stabilisation of the signal. Thus, 15 min was selected as the incubation time.

3.6 Analytical performance

Under the optimised experimental conditions described above, the determination of AZY molecules at different concentrations was evaluated by DPV (Fig. 7).

A linear relationship between the oxidation peak current and the concentration was exhibited in the range between 0.5 and 10.0 μM ($r = 0.9963$, $n = 7$). The limits of detection (LOD) and quantification (LOQ), given by the equations $\text{LOD} = 3s/m$ and $\text{LOQ} = 10s/m$, where "s" is the standard deviation of the intercept and "m" is the slope of the calibration plot, are found to be 0.08 and 0.3 μM , respectively.

The sensitivity of the method is appropriate for monitoring AZY in environmental water samples given that these limits are obtained without any sample pre-treatment (solid phase extraction, SPE) which can concentrate 100 $\times$ to 1000 $\times$ the level of contamination depending on the water origin (wastewater,

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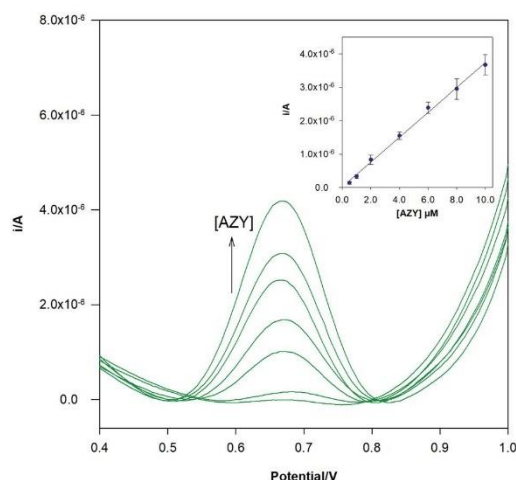
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Fig. 7 DPV voltammograms obtained for MIP sensor analysis with different AZY concentrations. Inset: linear relationship between peak current intensity and AZY concentration in the range 0.5 to 10.0 μM .

tap, river).^{49,50} There are other electrochemical MIP-sensors proposed for detection of AZY by incorporation of nano-materials⁵¹ or using electrochemiluminescence⁵² with lower detection limits. However, the proposed sensor is simple, easy to prepare and disposable, which make it appropriate for future commercial applications.

The repeatability (intra-day precision) and reproducibility (inter-day precisions) of the MIP-sensor were estimated at two levels of concentration (2 and 8 μM) and expressed as relative standard deviation (RSD). Four analyses per day were performed on four different days.

The repeatability was found to be 5.9 and 7.1% for a concentration of 2 and 8 μM , respectively, while the reproducibility was 9.3 and 11.6% for the same concentrations. These results demonstrate that the MIP-sensor has satisfactory levels of precision, both for intra-day and inter-day analysis.

Finally, the stability of the MIP sensor was also evaluated. The as-prepared MIP-sensor was stored dry at room temperature, and its response was evaluated after 15 and 30 days. It was found that the sensor response corresponds to 86% and 82% of the initial signal after it has been stored for 15 and 30 days, respectively.

3.7 Selectivity of the sensor

To evaluate the selectivity of the developed sensor, four analogues of AZY were selected, CLY, ERY, STRE and TETRA, and prepared at a concentration 10-fold the AZY concentration. Both NIP and MIP sensors were used to analyse solutions of each individual compound. The results were expressed as % of the peak current relative to the MIP peak current obtained for AZY (Fig. 8). As expected, the experimental results clearly demonstrated that the response signal of AZY after incubation on the MIP sensor was higher than those of other macrolides,

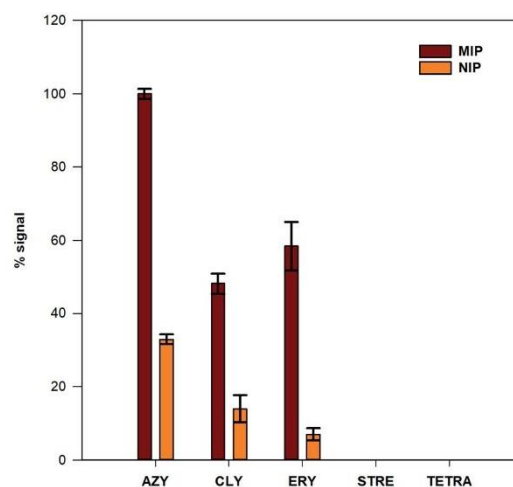


Fig. 8 Selectivity studies.

indicating the effective imprinting process. The responses to CLY and ERY, even though they closely resemble the chemical structure of AZY, were much lower with oxidation peaks corresponding to 48.1 and 58.4% of the AZY peak, respectively. This could lead to a conclusion that one more dialkylamine group present in AZY is responsible for enhancing the hydrogen-bonding affinity between AZY and the imprinted cavities on the MIP. Finally, when STRE and TETRA solutions were analysed, no response was observed. Therefore, the developed MIP sensor exhibits a selective recognition ability toward AZY molecules.

3.8 Application to water samples

Finally, the application of the MIP sensor for AZY determination in real samples was performed using the standard addition method, without any pre-treatment steps. Tap water and water collected upstream of a WWTP output in the Ave River were spiked with two different concentrations (0.5 and 4 μM) of AZY. The recoveries were found to be in the range between 84 and 123% (Table 2). The results show that the proposed MIP-sensor

Table 2 Determination of AZY concentration in spiked water samples with the developed MIP sensor^a

Sample	[AZY]add/ μM	[AZY]det/ μM	Recovery/%
River water	0	—	—
	0.50	0.59	118
	4.0	4.92	123
Tap water	0	—	—
	0.50	0.42	84
	4.0	3.82	96

^a [AZY]add: AZY concentration added to the sample; [AZY]det: AZY concentration determined in the sample, using the MIP sensor developed in this work.

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could be used as an accurate and cheap green strategy, for determination of AZY in water samples without the need for laborious pre-treatment steps or methods.

4. Conclusions

A selective and rapid MIP electrochemical sensor, for the detection of AZY in water samples, was developed in this work. Functional monomer screening was performed using computational models, which allowed a faster and environmentally friendly approach. The prepared sensor exhibited a good linear relationship ($r = 0.9963$) in the concentration range of 0.5–10.0 μM with a low LOD (0.08 μM). The sensor showed a good selectivity in the presence of other structurally analogous compounds. Furthermore, the results of the analysis of tap water and water collected upstream of a WWTP output in the Ave River, with satisfactory levels of precision, indicate that the constructed MIP sensor may be a promising green technique for monitoring AZY in environmental samples.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Patrícia Rebelo is grateful to FCT (Fundação para a Ciência e a Tecnologia) for her PhD grant (SFRH/BD/132384/2017). João Pacheco is grateful to FCT for his postdoc grant (SFRH/BPD/101419/2014), financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu and Ministério da Ciência, Tecnologia e Ensino Superior. This work was further supported by UID/QUI/50006/2019 with funding from FCT/MCTES through national funds. The authors would like to thank the EU and FCT/UEFISCDI/FORMAS for funding, in the frame of the collaborative international consortium REWATER financed under the ERA-NET Cofund WaterWorks 2015 Call. This ERA-NET is an integral part of the 2016 Joint Activities developed by the Water Challenges for a Changing World Joint Programme Initiative (Water JPI). The authors would like to thank the project Farmasense (39957) that was funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020) and the Fundo Europeu de Desenvolvimento Regional (FEDER).

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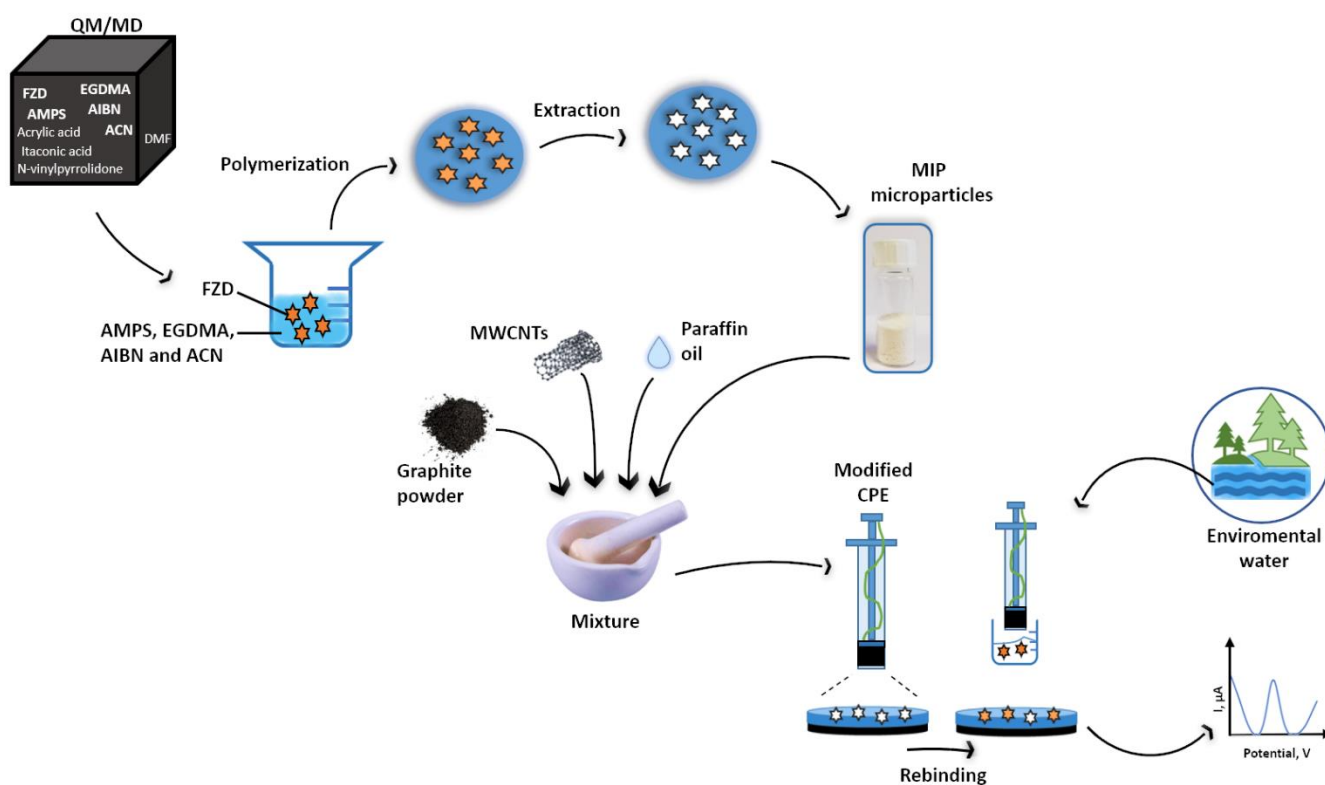
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3.2 Rational development of molecular imprinted carbon paste electrode for furazolidone detection: theoretical and experimental approach

Sensors and Actuators: B. Chemical 329 (2021) 129112



Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review, investigation, methodology and writing of the original draft.

Highlights

- First time a selective MIP electrochemical sensor was developed for Furazolidone (FZD).
- QM calculations and MD simulations were used to select the best functional monomer and the optimum polymerization conditions.
- The sensor constructed based on MIP-CPE-MWCNTs showed high selectivity and sensitivity.
- The proposed sensor was successfully applied for FZD detection in water samples without any pre-treatment step.



Rational development of molecular imprinted carbon paste electrode for Furazolidone detection: theoretical and experimental approach

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ARTICLE INFO

Keywords:

Molecularly imprinted polymer
Computational design
Environmental monitoring
Electrochemical sensor
Voltammetry

ABSTRACT

Determination of antibiotics in environmental waters is an important global issue. Although furazolidone (FZD) was banned from use in food-producing animals, owing to its mutagenic and carcinogenic effects, this antibiotic has been illegally used across the world and its presence in environment have being noted.

In this work, the first selective molecularly imprinted polymer (MIP) was developed for electrochemical detection of FZD. It was constructed based on the modification of the traditional carbon paste electrode (CPE) with MIP microparticles, followed by introduction of multi-walled carbon nanotubes (MWCNTs). Quantum mechanical (QM) calculations and molecular dynamics (MD) simulations were performed to allow rational selection of an appropriate functional monomer and to simulate the best pre-polymerisation conditions, respectively. The MIP were synthesized by polymerization using 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) as monomer and FZD as template molecule. The MIP microparticles were then incorporated on CPE-MWCNTs and the electrochemical analysis of FZD were evaluated by differential pulse voltammetry (DPV). After optimisation of experimental conditions, the MIP-CPE-MWCNTs sensor exhibited a good linear response over the concentration range of 0.01 μM to 1 μM with a correlation coefficient of 0.9995. The limit of detection (LOD) was found to be 0.03 μM ($S/N = 3$). Due to high imprinting efficiency the sensor displayed selectivity to recognise FZD molecules and it was successfully applied in water samples where excellent recovery values (over 90 %) were obtained. The proposed sensor provides an efficient and promising sustainable strategy for monitoring of FZD in environmental waters.

1. Introduction

Furazolidone (FZD) is a broad-spectrum nitrofurantoin antibiotic that is commonly used to prevent and treat bacterial diseases in humans and animals [1]. Due to low production cost and high effectiveness, it has also been reported to be used as growth promoter in farm animals and aquaculture [2,3]. However, its potential toxicity and carcinogenicity has raised human health concern around the world [4,5].

In 1995, the use of FZD in food-producing animals was prohibited in Europe by the European Commission and years later the ban was extended to the United States and other developed countries [6,7]. As a number of authors highlighted, this antibiotic can be rapidly metabolised to its metabolites such as 3-amino-2-oxazolidinone and β -hydroxyethylhydrazine, which biochemical toxicity leads to inhibition of monoamine oxidase [6,8]. Despite prohibition, FZD is still illegally

administered with inappropriate dosage mainly in livestock farms mixed with animal feed or drinking water [9]. Since FZD is known to have very low assimilation efficiency [10], it is accidentally introduced into the environment and its presence in water bodies has been reported [11,12]. While there are no developed studies showing a clear and unequivocal conclusion about adverse effects in humans, its toxicity to various aquatic organisms has been described [6]. Moreover, the high possibility of the escape of FZD into the environment contributes to increase global antibiotic contamination with evolution and spread of antibiotic resistance as one of the most worrying consequences [13,14].

Antibiotics persist into environmental waters and, even at low concentration, can directly or via long-term exposure affect the survival and behaviour of many species [15,16]. Therefore, the development of specific, simple, and rapid analytical tools to screen antibiotic levels in aquatic environment is extremely important and necessary.

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<https://doi.org/10.1016/j.snb.2020.129112>

Received 25 July 2020; Received in revised form 22 October 2020; Accepted 23 October 2020

Available online 28 October 2020

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Among different techniques reported for monitoring FZD, including high-performance liquid chromatography (HPLC) [17], liquid chromatography-mass spectrometry (LC-MS) [5], enzyme-linked immunosorbent assay (ELISA) [18], lateral flow immunoassay (LFIA) [19] and spectrometry [20], electrochemical sensors have recently attracted increasing attention [2,21,22]. By comparison with the other mentioned methods, electrochemical sensors provide attractive properties such as simplicity, low cost, versatility, and faster responses. In the last few decades, significant progress has also been made in LFIA tests [23] and, due to their remarkable properties, such as fast, low cost, portable and easy-to-use assay, they are considered an ideal choice for point of care applications [24,25]. However, the device behind the LFIA has a complex architecture with many variables that have to be controlled to ensure reproducible data processing. The choice of the best antigen or antibody and reagent stability are the most critical steps [23, 25]. In addition, the results are mostly qualitative or semi-quantitative and the sensitivity and selectivity are variable [24,26]. Selectivity is an essential requirement for electrochemical biosensing and, in this context, molecularly imprinted polymer (MIP) has become a well-established approach thanks to unique properties like high specificity towards the target analyte and high chemical and physical stability [27]. MIP based sensors have been successfully employed as an alternative strategy for pharmaceuticals detection in environmental waters [28,29]. Interestingly, their combination with conductive nanomaterials such as multiwalled carbon nanotubes (MWCNTs) allows for greatly improved sensitivity and overcoming the lack of conductivity and electrocatalytic activity [30]. Because of their large surface area and easy functionalisation, MWCNTs are also widely used as the supporting material to achieve accelerate electron transfer on MIPs surface [31].

One of the most important and essential steps in MIPs construction are the selection of the suitable functional monomer for each desired target compound (so-called template), as well as its ratio to template in the polymerisation solution. These experimental steps usually involve the use of significant amounts of chemicals and solvents and, consequently, time- and resource-consuming work [32]. Nowadays, efforts have been made to develop new strategies to surmount these drawbacks where theoretical approaches have shown to be a good initial solution. The use of computational screening methods such as quantum mechanical (QM) calculations and molecular dynamics (MD) simulations, applied for rational design of MIPs, has been reported [33,34]. QM allows to understand interactions between a template and virtual libraries of functional monomers via energy minimisation calculations [35]. At the same time, MD simulations is a great tool to predict physical and chemical properties. By simulating the MIP pre-polymerisation conditions, it is possible to infer interactions between the functional monomer and specific template monomer [35]. Despite the advantages of computational studies, few investigations have been performed so far to predict MIP optimal conditions before experimental tasks.

In the present work, we report for the first time an electrochemical device based on MIP modified carbon paste electrode (CPE) for FZD detection. The sensor was constructed by traditional precipitation polymerisation after preliminary tests using QM and MD computational calculations. With the obtained insights from the latter, the best functional monomer was chosen, and the polymerisation system composition was tested experimentally and proved its accuracy. Therefore, taking the advantages of MWCNTs signal amplification the sensitivity of analysis was improved and a highly selective MIP-CPE-MWCNTs sensor for electrochemical determination of FZD was developed. Finally, the new electrochemical sensor was successfully applied for determination of FZD in spiked water samples.

2. Methods

2.1. Reagents

FZD was obtained from Sigma-Aldrich. The standard solution was

prepared by dissolving an appropriate amount of the antibiotic in acetonitrile (ACN) and directly diluting in phosphate buffer (0.1 M, pH = 7). Working solutions were prepared daily and stored in absence of light until use. 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPS, 99 %), ethylene glycol dimethacrylate (EGDMA, 98 %), 2,2'-azobisisobutyronitrile (AIBN, ≥ 98 %), multiwalled carbon nanotubes (MWCNTs, $> 8\%$ carboxylic acid functionalized), analogues of FZD (nitrofurantoin (NTF) nitrofurazone (NFZ) and furaltadone (FTD)), *N,N*-dimethylformamide (DMF, ≥ 98 %) were also purchased from Sigma-Aldrich. Methanol (MeOH, ≥ 99.9 %) and paraffin oil were obtained from Merck. Spectroscopic carbon graphite powder (Ultra Carbon, Ultra F Purity) was purchased from Mersen. Chromatographic grade ACN, acetic acid glacial, ethanol, sulfuric acid (H_2SO_4 , 96 %) were supplied by Carlo Erba. All chemicals were used as supplied without any further purification. Ultra-pure water (resistivity = $18.2 \text{ M}\Omega \cdot \text{cm}$) obtained from a Millipore (Simplicity 185) water purification system was utilised in all experiments. Phosphate buffer solution (0.1 M, pH = 7) was prepared with KH_2PO_4 and K_2HPO_4 (Riedel-de-Haën). The water samples were collected from tap and upstream of the wastewater treatment plant (WWTP) output in Lis river in Leiria.

2.2. Apparatus

Differential pulse voltammetry (DPV) was conducted at room temperature using a Metrohm Autolab PGSTAT 204 potentiostat/galvanostat controlled by NOVA 1.10 software. A typical three-electrode system was used for all measurements: the constructed MIP-CPE-MWCNTs acts as a working electrode, a platinum wire and a KCl-saturated Ag|AgCl electrode as the auxiliary and reference electrodes, respectively. Scanning electron microscope (SEM) images were acquired by FEI Quanta 400 FEG ESEM/EDAX Pegasus X4M equipment at CEMUP (Centro de Materiais da Universidade do Porto).

2.3. Computational design

2.3.1. QM setup

Eleven different functional monomers including AMPS, *N*-vinylpyrrolidone, itaconic acid, methyl methacrylate, 4-vinyl pyridine, methacrylic acid, acrylic acid, 4-ethylstyrene, styrene, 1-vinylimidazole and 2-vinyl pyridine, were studied as potential monomers for FZD-MIP film formation. The VMD software [36] was used to build all the molecular fragments. Their geometric configurations were pre-optimised with the aid of Gaussian 09 program [37] at a PM6 or a PDDG semi-empirical level. The template molecule was then combined with functional monomers to generate the respective complexes, using the program AutoDock [38] within the VMD plugin VsLab [39]. Calculations of the lowest energy structures corresponding to the best complex conformation were carried out using the M06-2X/6-31+G* density functional theory (DFT) level, which allows a better description of the dominant dispersive interactions in molecular systems. The binding energies (ΔE) were finally calculated by equation (Eq (1)):

$$\Delta E = E(\text{complex}) - E(\text{template}) - E(\text{monomer}),$$

where $E(\text{complex})$ represents the energy of the monomer-template complex, $E(\text{template})$ the energy of the template and $E(\text{monomer})$ the energy of the monomer.

2.3.2. MD simulations

All MD simulations were carried out with the GROMACS 5.1.4 software package [40]. The studied pre-polymerisation mixtures comprised varying number of molecules (see section 3.2, Table S1). The General Amber Force Field (GAFF) was used for all molecular species as received from module ANTECHAMBER [41] with the exception of AMPS molecule. Generally, GAFF presents a good performance in simulation of MIPs [42,43], featuring the stability of hydrogen bonding (H-bonding)

and van der Waals adducts. However, for AMPS the force field (FF) had to be modified considering the inability of GAFF to correctly reproduce the geometry of the molecule. The intramolecular potentials for bonds, angles and dihedral angles for the $-\text{CSO}_3\text{H}$ fragment of AMPS were taken from the literature [44]. Partial atomic charges for all mixture components were assigned using the ANTECHAMBER program.

The initial configurations were obtained by randomly placing the mixture components into cubic simulation cells with the PACKMOL code [45]. First, an energy minimisation was performed with the steepest-descent algorithm to full relaxation of each system (a maximum force tolerance of $10 \text{ kJ mol}^{-1} \text{ nm}^{-1}$ was used). Second, two pre-equilibrations of 100 and 500 ps each were carried out at 298.15 K in canonical and isothermal-isobaric ensembles, respectively. Then, an additional relaxation by annealing followed: the systems were heat up to 350 K for 400 ps, maintained at this temperature for 200 ps, and then slowly cooled back to 298.15 K for the next 400 ps. This stage allowed the pre-polymerisation system to cross over possible energy barriers. The step size of 0.5 fs was employed for all the described above simulations steps. No bonds were constrained during this first phase of modelling. Next, the equilibration of the systems in the NPT ensemble was carried out for 1 ns with 1 fs time step. At this stage of the simulation, the bonds with H-atoms were constrained with the LINCS algorithm [46]. Finally, a production run in the NVT ensemble for 20 ns was carried out. Throughout the simulations, the temperature was maintained constant with the assistance of the Nose-Hoover thermostat [47, 48] (with coupling constant of 2.0 ps) and pressure when needed was kept at 1 bar with the Parrinello-Rahman barostat [49] (with coupling constant of 8.0 ps). The equations of motion were integrated with the leap-frog algorithm [50]. The long-range electrostatic forces were treated by the particle-mesh Ewald method [51] and a cut-off of 1.2 nm was applied. The same cut-off was employed for the short-range interactions and neighbour lists. Long-range van der Waals interactions were treated using a continuum model correction for energy or energy and pressure, according to the ensemble used. Periodic boundary conditions were applied along all Cartesian directions. The in-built GRO-MACS tools were used for the calculations of density, self-diffusion coefficients, assessment of radial distribution functions (RDFs), and analysis of H-bonds and clusters. The numerical integration of RDFs for coordination numbers (CNs) assessment was performed using the GRACE software.

2.4. Preparation of MIP by precipitation polymerisation

In a glass tube, 0.2 mmol FZD and 1.2 mmol AMPS were first dissolved into 20 mL of ACN and stirred for 1 h to allow self-assembly of the pre-polymerisation complex. Then, 4 mmol EGDMA and 0.6 mmol AIBN were added and the mixture, with a molar ratio between template: monomer:crosslinker of 1:6:20, was purged with nitrogen during 10 min. Subsequently, the tube was tightly sealed and kept in the thermo block (Temblock, Selecta®, Spain) at 65°C for 24 h. The obtained polymer was washed with ACN to eliminate the unbound polymer and with a mixture of acetic acid-MeOH (1:9, v/v) successively until no voltammetric FZD signal was observed, followed by centrifugation to remove solvent. Finally, the polymeric microparticles were washed three times with ethanol and dried in an oven at 60°C . The non-imprinted polymer (NIP) was also prepared using the same procedure but in the absence of FZD.

2.5. Construction of MIP modified CPes-MWCNTs

To fabricate MIP and NIP modified CPes, an appropriate amount of graphite powder, polymeric microparticles of MIP/NIP and MWCNTs were thoroughly mixed using a mortar and pestle for at least 5 min to provide a homogeneous mixture. After blending, paraffin oil was added, and the mixture was again mixed for 10 more minutes. The resulting uniform paste was packed at the end of a syringe with an internal

diameter of 4 mm and a copper wire for electrical contact. Then, the paste was compressed by the piston and polished on a paper sheet to form a smooth and reproducible working surface. At the end of each measurement, the paste was replaced, following the same procedure of compression and polishing.

2.6. Electrochemical measurements

Electrochemical measurements for the determination of FZD were performed by differential pulse voltammetry (DPV). Firstly, the prepared electrodes were immersed in FZD solutions for 30 min and carefully washed with distilled water and put in phosphate buffer during 10 s to avoid residues of FZD. Then, the electrodes were inserted into an electrochemical cell containing 0.1 M H_2SO_4 as supporting electrolyte. DPV experiments were conducted in the potential interval from $+0.2 \text{ V}$ to -0.8 V using a pulse amplitude of 50 mV and a step potential of 0.5 mV. All measurements were carried out in triplicate and at room temperature.

2.7. Sample preparation

Two different water samples were selected: tap water and Lis river water collected upstream of WWTP in Leiria. They were directly used as real samples without pre-treatment.

3. Results and discussion

3.1. Theoretical selection of a suitable monomer

Aiming to obtain the higher binding capacity between FZD and functional monomer, computer calculations were performed as a previous step to the FZD-MIP preparation. Based on the most stable conformational structure, *i.e.* the structure presenting the lowest energy, DFT calculations were applied to estimate the binding energies between the complex formed by FZD molecule and each tested monomer selected. The results, listed in Table 1, show the binding energies of FZD-monomer complexes in vacuum with mole ratios of 1:1, obtained through Eq (1). Generally, polymer complexes with higher absolute interaction energies are more stable and, consequently, are expected to be the most suitable for preparation of MIPs [52]. As it is shown in Table 1, FZD interacts most strongly with the AMPS monomer giving rise to a complex binding energy of $-71.943 \text{ kJ mol}^{-1}$, followed by N-vinylpyrrolidone and itaconic acid. These results suggest that AMPS is the best candidate for molecular imprinting. Thus, AMPS was chosen as functional monomer for the further experiments. The optimised geometries of FZD, AMPS, EGDMA, AIBN, ACN and DMF with the designation of important interactions sites used throughout the work are shown in Fig. 1.

Table 1
Binding energies of FZD-monomer complexes obtained at M06-2X/6-31+G* level.

Complex	$\Delta E/\text{kJ mol}^{-1}$
FZD-(AMPS)	-71.943
FZD-(N-vinylpyrrolidone)	-56.356
FZD-(Itaconic acid)	-56.084
FZD-(Methyl methacrylate)	-54.669
FZD-(4-vinylpyridine)	-51.632
FZD-(Methacrylic acid)	-50.629
FZD-(Acrylic acid)	-49.988
FZD-(4-ethylstyrene)	-46.146
FZD-(Styrene)	-38.577
FZD-(1-vinylimidazole)	-29.113
FZD-(2-vinylpyridine)	-26.854

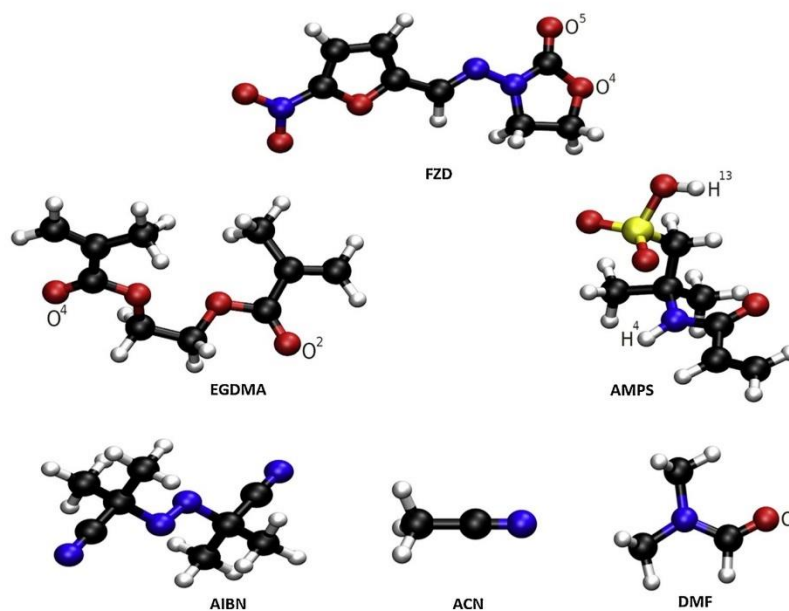


Fig. 1. Optimised geometry and interaction sites of the studied molecules: furazolidone (FZD), 2-acrylamido-2-methylpropane sulfonic acid (AMPS), ethylene glycol dimethacrylate (EGDMA), 2,2'-azobisisobutyronitrile (AIBN), acetonitrile (ACN) and *N,N* dimethylformamide (DMF).

3.2. MD simulation of pre-polymerisation mixtures

The choice of an appropriate functional monomer is a crucial step to obtain MIPs with desirable recognition characteristics but fine-tuning of other characteristics is also mandatory. MIP properties are additionally determined by suitable molar ratio of template/monomer/cross-linker during the polymerisation reaction [53]. Therefore, these variables should be considered when preparing MIPs. In the case of non-covalent imprinting, functional monomers are usually added in the molar excess to the number of moles of the template in order to favour the formation of the monomer-template complex [54]. On the other hand, specific interactions can be reduced by using extra amounts of monomers [55]. In most of the works reported to date, the identification of the optimum amount of these components is rarely carried out and a typical template-monomer molar ratio of 1:4 is used as standard [56]. Taking this into account, six different pre-polymerisation systems were tested by MD simulations with different molar ratios of template (FZD) to functional monomer (AMPS). The systems composition is summarised in supporting information, Table S1. All systems were prepared using the same cross-linker (EGDMA), initiator (AIBN) and the same solvent stoichiometry. In order to investigate the contribution of porogenic solvent on the polymer performance two solvents were selected. ACN was used in simulation of five systems (systems from A to E) and, based on system C stoichiometry, DMF was chosen for system F.

3.2.1. Density and self-diffusion

As a first step of the simulation, the validation of ACN and DMF FF models were performed. For this purpose, a short additional MD run for pure ACN and DMF (2000 molecules in the cell, 1.5 ns run) were carried out. The obtained values of density ($790.9 \pm 0.4 \text{ kg m}^{-3}$ for ACN and $973.5 \pm 0.2 \text{ kg m}^{-3}$ for DMF) and self-diffusion coefficients ($2.30 \pm 0.03 \times 10^{-9}$ and $0.90 \pm 0.02 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ for ACN and DMF, respectively) are consistent with the reported in literature experimental ones [57–60]. Thus, the used FFs describe properly the solvents under study and can be applied for further simulation. The quality of the simulations of the pre-polymerisation mixtures was controlled by the

density and self-diffusion coefficients.

As it can be seen in Table S2, a good agreement was found between the concentration and density. Indeed, the results show increase in the systems density with the increase of concentration of AMPS, as expected. Self-diffusion of FZD in the systems with ACN slightly increases with the rise of AMPS content as well and the highest value is obtained in system D. As for the influence of the solvent, the diffusion values of all species reach the lowest values in the system with DMF (System F), which highlights the importance of porogen in the pre-polymerisation mixture [61]. Taking into account that mobility of FZD is important to allow its diffusion in the binding sites [62], system D seems to be the most promising system in rebinding processes. At the same time, the values of the diffusion coefficients to AMPS declined from system A to system F, which can suggest that a dense polymer network retards the diffusion of functional monomer in solution. A similar behaviour was observed for EGDMA, AIBN and ACN.

3.2.2. Local structure: RDFs and CNs

RDFs with respective CNs were accessed to study local interactions between the main components of the imprinting process at the molecular level. The distribution of functional monomer around the template molecule is one of the most important factors in the molecular imprinting process. In this step, cross-linkers have an important role in controlling the morphology of polymer matrix, since they fix functional monomers to templates and, consequently, affect the rigidity of the polymer network formed [53]. In the studied systems, the interactions between specific parts of the molecules were evaluated and only the most active sites were selected for the discussion. The RDFs reported in Fig. 2 showed favourable interactions between FZD and AMPS, as well as between AMPS and EGDMA, indicating a good imprinting effect. Closer analysis revealed that FZD displays strong interactions by oxygen of the carbonyl group (O^5 , see Fig. 2), while the extent of interaction of AMPS is made by two hydrogen atoms (H^{13} and H^4 , see Fig. 2). In addition, it was found that two oxygen atoms of EGDMA (O^2 and O^4) have an important role in binding with AMPS. $\text{O}^5_{\text{FZD}}\text{H}^4_{\text{AMPS}}$, $\text{O}^5_{\text{FZD}}\text{H}^{13}_{\text{AMPS}}$, $\text{H}^4_{\text{AMPS}}\text{O}^{2,4}_{\text{EGDMA}}$ and $\text{H}^{13}_{\text{AMPS}}\text{O}^{2,4}_{\text{EGDMA}}$ RDFs exhibit well defined

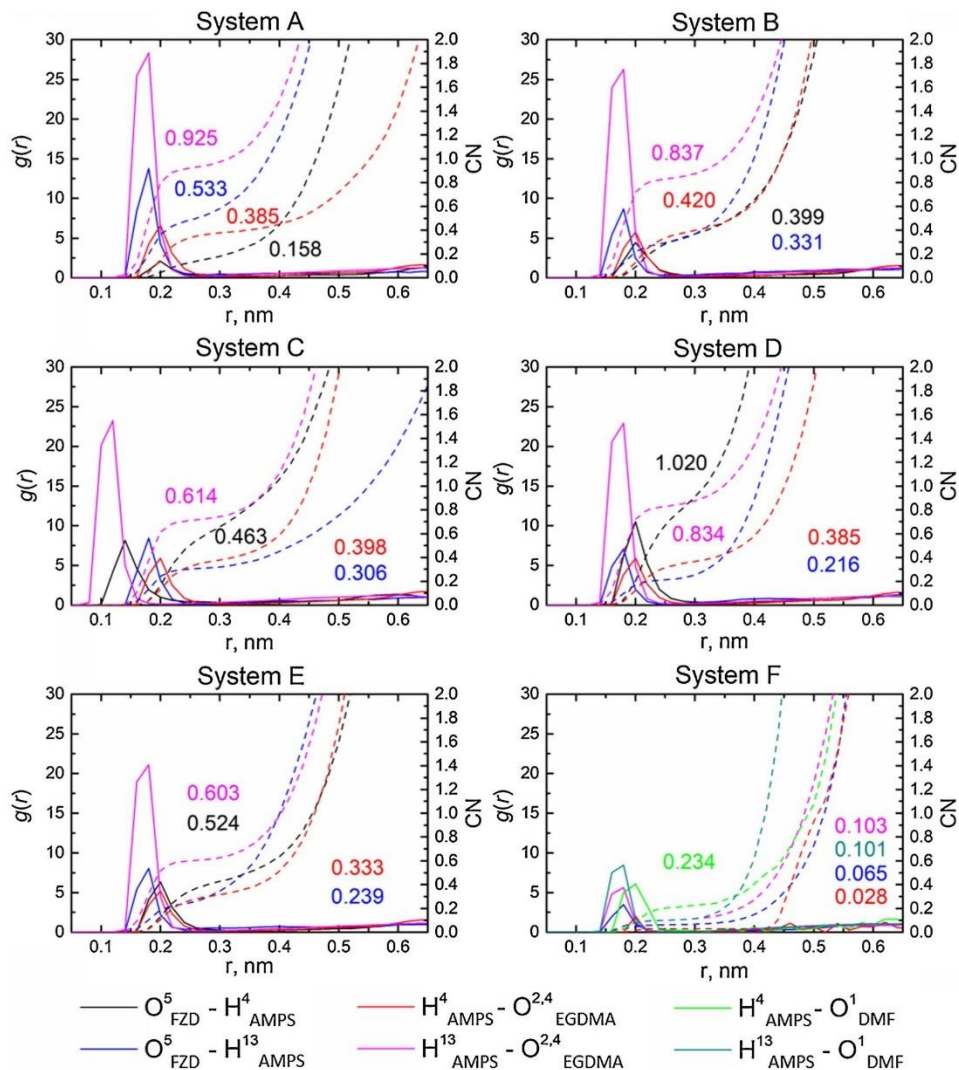


Fig. 2. Radial distribution functions, $g(r)$, (left axis, solid lines) between specific sites of FZD, AMPS, EGDMA and DMF molecules and corresponding coordination numbers, CNs, (right axis, dash lines) in the studied pre-polymerisation systems from the results of molecular dynamics simulations. Numbers represent CNs according to colour code in the legend.

peaks at about 0.2 nm, which suggests the presence of H-bonds. Using ACN as solvent (System A–E), a high and narrow peak was observed between H_{AMPS}^{13} and $O_{EGDMA}^{2,4}$ in all systems, showing the stability and strength of this contact even upon AMPS concentration change. In contrast, significant differences were observed in the height of the peaks of $O_{FZD}^5 - H_{AMPS}^4$ and $O_{FZD}^5 - H_{AMPS}^{13}$. The interaction between FZD and AMPS molecules is expected to be enhanced by increasing the concentration of AMPS. In fact, there are two high peaks corresponding to $O_{FZD}^5 - H_{AMPS}^4$ and $O_{FZD}^5 - H_{AMPS}^{13}$ interactions at short distance in systems C and D, which clearly confirm the affinity. The height of these peaks slightly decreased in system E. On the other hand, RDFs analysis of system F showed a great interference of AMPS with oxygen (O^1) of the carbonyl group of DMF. This undesirable interaction at close distance seems to impair the aggregation of AMPS with other components of the

system. Thus, interactions of AMPS with EGDMA and FZD are comparatively less favourable and lower peak heights were observed.

These results are further confirmed by the coordination numbers' analysis. Notice, that to obtain the CNs, the RDF were integrated up to the first minimum on the corresponding interaction. In system D, FZD-AMPS and AMPS-EGDMA interactions have the highest total CNs (1.236 for $O_{FZD}^5 - H_{AMPS}^{4/13}$ and 1.219 for $H_{AMPS}^{4/13} - O_{EGDMA}^{2,4}$) (Fig. 2), suggesting that conditions of system D appear to be more susceptible to form favourable interactions within the pre-polymerisation mixture.

3.2.3. H-bonding

Considering the RDF results, an analysis of the extent and stability of H-bonds was performed paying special attention towards the surrounding environment of O_{FZD}^5 , H_{AMPS}^4 , H_{AMPS}^{13} and $O_{EGDMA}^{2,4}$ sites. In

this context, the H-bonding network was analysed following the framework of Luzar and Chandler geometrical criterion [63], which implies that the distance between the oxygens of two different molecules is less than 3.5 Å and, simultaneously, the angle formed by O-H...O is less than 30°. The occurrence and lifetime of the H-bonds extracted in this screening process are summarised in Table 2. As it can be noticed, the total amount of H-bonds between FZD and AMPS clearly increases from system A to system D being virtually the same in systems D and E. This can be regarded as a sort of saturation of FZD-AMPS interaction capacity at a given FZD content. The number of contacts between AMPS and EGDMA species consistently increases with the increased molar fraction of functional monomers from system A to E. These findings are in line with the described above results of RDFs and CNs analyses. Due to the fact, that the recognition during the complex polymerisation is mainly based on hydrogen bond formation, it is assumed that the maximum number of hydrogen bonds with the template corresponds to the optimum imprinting factor [64]. Consequently, the importance of the stoichiometry of system D for imprinting quality was again highlighted. Besides the extent of H-bonding interaction FZD-AMPS is similar in systems D and E, the associated average weighted lifetimes suggest that bindings in system D (with a lifetime of 35.54 ps) are more stable (longest average lifetimes) and potentially create higher affinity binding sites. Noteworthy, the hydrogen bond capacity was strongly reduced by changing the solvent from ACN to DMF (System F). This can be explained by the interaction of porogen with the complex, which then competes with the binding mechanism of the FZD-AMPS interactions.

3.2.4. Cluster size analysis

The ability of FZD, AMPS and EGDMA to assemble in multi-molecular aggregates (*i.e.* clusters) is the next issue to be addressed in all studied systems. The FZD-AMPS clustering behaviour can be easily accessed by means of MD simulations and it is of primary importance. The first minimum on FZD-AMPS RDFs between the most active interaction sites was used as distance criteria for analysis of clusters (in minimum, the cut-off distances for different RDFs range from 0.28 to 0.32 nm, see Fig. 2). The average and maximum cluster sizes in the simulated systems provide the more accurate representation of the FZD-AMPS interaction, as it can be seen in Table 3. The largest FZD-AMPS clusters are formed in systems D and E, which is in accordance with their hydrogen bonding capacity. Once again, the change in cluster size tends to stabilise after system D. The geometries of typical FZD-AMPS clusters are shown in Fig. 3. Another important observation is a systematic cluster size drop observed in the simulation of System F. The average FZD-AMPS cluster size decreased to 1.14, meanwhile the maximum cluster size decreased to 6 molecules in the presence of DMF. This decrease of the aggregate size may indicate that there are less FZD and AMPS molecules, not screened by DMF and therefore available for participation in this binding.

To sum up, system D exhibits the most appropriate behaviour of key structural and dynamics properties, which makes it the most promising pre-polymerisation mixture for the MIP development. Indeed, it provides a good diffusion of FZD within the polymer pores and strong and closer interactions with hydrogen atoms of AMPS (H^{13} and H^6). Moreover, H-bonding analysis of the simulations, besides confirming the importance of these interactions, demonstrated their good stability.

Table 2

Average number of hydrogen bonds per frame and their lifetimes (in ps in parenthesis).

Pairs		System A	System B	System C	System D	System E	System F
FZD-AMPS	O^5-H^4	0.02 (7.94)	0.08 (6.36)	0.31 (6.80)	0.55 (8.86)	0.45 (7.42)	—
	O^5-H^{13}	0.09 (19.68)	0.11 (25.95)	0.21 (15.73)	0.26 (26.68)	0.39 (23.85)	0.07 (20.65)
	Total	0.11	0.19	0.52	0.82	0.84	0.07
AMPS-EGDMA	$H^4-O^{2,4}$	2.45 (9.60)	4.25 (10.61)	8.41 (12.13)	12.50 (11.82)	14.42 (11.51)	2.61 (10.78)
	$H^{13}-O^{2,4}$	7.67 (43.36)	10.04 (43.34)	24.21 (41.58)	35.21 (43.88)	42.75 (46.04)	4.45 (41.96)
	Total	10.12	18.29	32.62	47.71	57.17	7.06

Table 3

Average and maximum FZD-AMPS clusters sizes at different pre-polymerisation compositions.

Systems	Average cluster size	Maximum cluster size
A	1.15	5
B	1.24	10
C	1.45	15
D	1.54	18
E	1.56	18
F	1.14	6

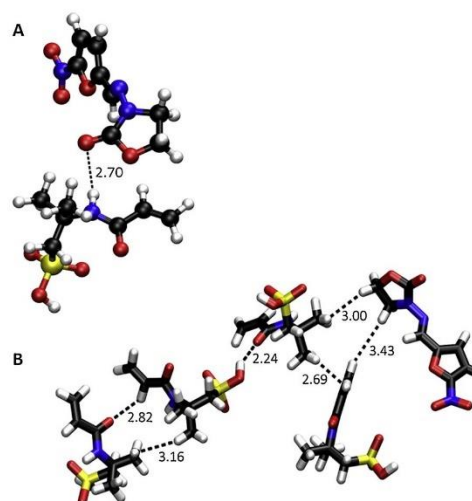


Fig. 3. Typical geometry of FZD-AMPS cluster in system D: (A) 1 FZD : 1 AMPS; (B) 1 FZD : 4 AMPS.

Finally, the analysis of clusters suggested the highest aggregation for FZD-AMPS species. Considering the poor performance of the system F, one can see that it does not fulfil the criterion of polymerisation. It presents weak interactions for the main bindings in the system, namely FZD-AMPS and AMPS-EGDMA, showing potential to adversely affect the specificity of the synthesised MIP. So, the MIP ratio of FZD to AMPS of 1:6 was selected as the best ratio for the imprinting procedure.

3.3. Electrochemical behaviour of FZD

After the combination of MIP and NIP with CPE and MWCNTs, the FZD molecules recognition ability was tested. Preliminary DPVs experiments were performed in 0.1 M H_2SO_4 after 30 min of incubation in 0.01 mM of FZD solution. The voltammograms are shown in Fig. 4 for (a) CPE, (b) CPE-MWCNTs; (c) NIP-CPE (d) MIP-CPE, (e) NIP-CPE-MWCNTs and (f) MIP-CPE-MWCNTs. No redox peak was observed after incubation of FZD in the CPE. However, when MWCNTs were added into the CPE a small peak appeared at -0.25 V, indicating that CPE-MWCNTs had

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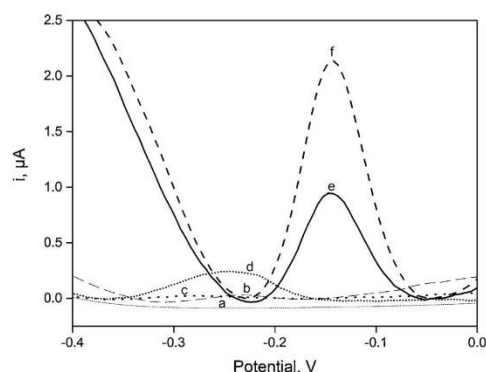


Fig. 4. DVP voltammograms in 0.1 M H_2SO_4 after 30 min of incubation in 0.01 mM FZD at a) CPE, (b) CPE-MWCNTs (c) NIP-CPE, (d) MIP-CPE, (e) NIP-CPE-MWCNTs and (f) MIP-CPE-MWCNTs.

higher response to FZD than CPE. This result can be attributed to the good electrochemical activity of the MWCNTs. A pronounced difference between the obtained peaks measured for MIPs and NIPs with and without MWCNTs can also be seen. For NIP-CPE and MIP-CPE an anodic peak was observed at about -0.25 V. By incorporation of MWCNTs, the anodic peak of FZD shifts to more positive potentials, indicating a more favourable oxidation process. Interestingly, the obtained peak current was found to be significantly higher in the presence of MWCNTs than in their absence, which supports the use of this nanomaterial in this work to enhance the detection signal of FZD. MWCNTs modification suggests an increase of surface area of electron conduction and, consequently, higher FZD molecules absorption. Noteworthy, the nonspecific binding of FZD to MWCNTs had no significant effect without polymer microparticles. The obtained results showed that the peak current response in the NIP- and MIP-CPE-MWCNTs was clearly higher comparing with the CPE-MWCNTs response. Despite the ability of AMPs to bind FZD molecules in the NIP-CPE-MWCNTs, these results indicate a lower binding specificity. In MIPs, the increase in the current response can be considered an evidence of the formation and properly functioning of the selective recognition sites. The difference between the intensity of electrochemical signal of the MIP and NIP proved the successful preparation of molecularly imprinted sensor under the conditions described above, where the amount of effective sites for FZD adsorption was elevated.

3.4. SEM characterisation

SEM analysis was performed to characterise the synthesized imprinted polymer microparticles. The Fig. 5 shows the SEM images of NIP (A) and MIP (B) polymer microparticles after polymerisation. As can be seen, NIP and MIP polymer display a porous structure with microparticles connected firmly together. However, there are significant differences between their particle size. MIP microparticles are bigger and more heterogeneous than NIP microparticles, indicating that the presence of FZD, which interacts with AMPs, exerts a strongly influence on the polymer morphology.

3.5. Optimisation of the MIP-CPE composition and experimental conditions

In order to obtain the optimal conditions for the determination of trace amounts of FZD, important parameters controlling the performance of the proposed sensor were optimised: (a) paraffin oil amount, (b) composition of MIP modified CPE, (c) MWCNTs amount and (d) incubation time. The measurements were performed in triplicate and the results are expressed in Fig. 6.

During the preparation of CPE, an appropriate amount of paraffin oil in the electrode composition was found to have a prominent role in the sensor response to FZD (Fig. 6 (A)). Under variation of the paraffin oil concentration (0.15, 0.30, 0.5 and 0.65 $\mu\text{L mg}^{-1}$), it was found that a minimum of 0.15 $\mu\text{L mg}^{-1}$ in relation to carbon paste is required to achieve a stable and compact paste to pack into the syringe. The current response of the electrode increased by increasing the paraffin content to 0.30 $\mu\text{L mg}^{-1}$. However, larger amounts of paraffin oil led to a remarkable downward trend, which can be the consequence of an increase in the electrode resistance due to the insulating effect of paraffin. Therefore, the optimum concentration of paraffin oil was established to be 0.30 $\mu\text{L mg}^{-1}$.

Then, keeping constant the paraffin oil amount in CPE, the content of MIP microparticles, ranging 2.5–20 % (w/w) was evaluated. As expected, increasing the electrode MIP content exhibited a significant enhancement in the electrochemical signal until a maximum at 10 % of the total amount of carbon (Fig. 6 (B)). This result is reasonable because incorporation of MIP provides the recognition sites at the electrode surface and, consequently, increases the electrode response to FZD molecules. On the other hand, a gradually descending response was observed with further increase of the MIP amount above the threshold value (10 % (w/w)), which may be attributed to electrode surface conductivity decreasing in the presence of bigger amount of MIP. This behaviour is in agreement with a number of reports for MIP composition in CPE [65–67].

Similar experiments were also carried out to improve the

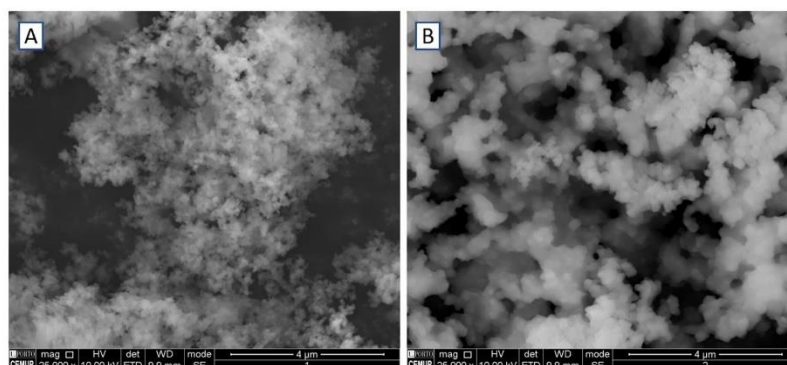


Fig. 5. SEM images of prepared (A) NIP and (B) MIP microparticles.

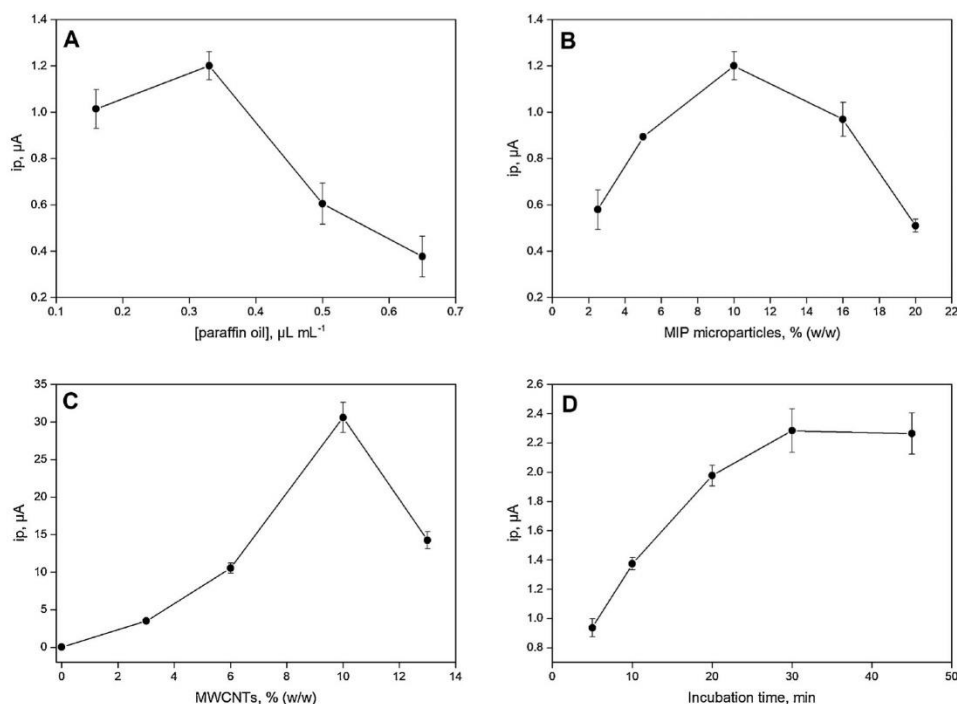


Fig. 6. Optimisation of carbon paste composition for determination of FZD at concentration of 10 μM. Variation of the peak current intensity with (A) the concentration of paraffin oil; (B) MIP nanoparticles content; (C) MWCNTs amount in percentage in carbon paste; (D) incubation time.

electrochemical properties of modified CPE. Different amounts of MWCNTs in the range of 3–13 % (w/w) were added to the carbon paste mixture including the optimum values of paraffin oil and MIP. The results present in Fig. 6 (C) showed that with the increase of MWCNTs there is a large increase of the peak current intensity. Generally, a larger effective surface area and stronger conductivity are reported in the presence of MWCNTs [68]. Likewise, in this work, the best electrochemical response in the MIP sensor as well was obtained when the MWCNTs (a total amount of 10 % for carbon paste) were added in CPE composition. Using higher percentage of MWCNTs, the negative effect on electrode response was observed. The fact that excessive amounts of MWCNTs are able to block the electron transfer process on the electrode surface can be a possible reason of this behaviour.

Another important parameter studied was the incubation time. For different times in the range of 5–45 min, the DPV responses of MIP-CPE-MWCNTs were recorded. This parameter demonstrated a substantial influence in the adsorption degree of FZD molecules. As it is clear from Fig. 6 (D), there is an increase in the peak current intensity with time until stabilisation after 30 min exposition, suggesting an incubation equilibrium on the MIP-CPE-MWCNTs. As longer incubation time did not show considerable effect on the FZD adsorption, 30 min therefore was selected as an appropriate incubation time.

Based on mentioned results, the CPE containing 0.30 μL mg⁻¹ of paraffin oil, 10 % of MIP and 10 % of MWCNTs showed the highest efficiency to absorb FZD molecules and it was selected for further experiments.

3.6. Analytical performance

Under the optimised experimental conditions, the MIP-CPE-MWCNTs sensor DPV responses towards different concentrations of

FZD were analysed (Fig. 7). The relationship between the oxidation peak intensity and the concentration exhibited a linear response in the range of 0.01 μM to 1 μM ($r = 0.9995$, $n = 3$). The limits of detection (LOD) and quantification (LOQ) were also calculated, based on $LOD = 3 s/m$ and $LOQ = 10 s/m$, where s is the standard deviation of the intercept and m is the slope of the calibration plot, and yielded 0.03 μM and 0.1 μM, respectively. In reproducibility measurements, four different electrodes were prepared with the same composition and used to determine 1 μM of FZD in solution. The relative standard deviation (RSD) result was 7.9 %, indicating a good precision and stable response of the proposed sensor.

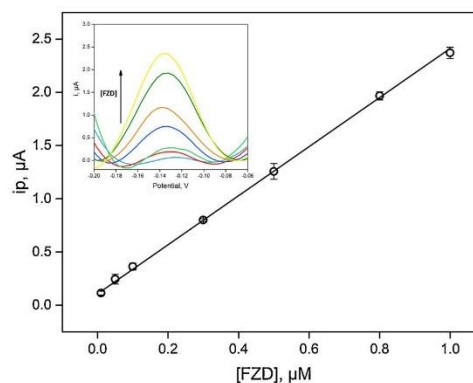


Fig. 7. Linear relationship between peak current intensity for MIP-CPE-MWCNTs and the concentration of FZD in the range 0.01–1 μM. The corresponding DPV voltammograms after incubation are shown in the inset.

3.7. Selectivity of MIP-CPE-MWCNTs

The selective recognition is one of the most important characteristics of a molecularly imprinted sensor. Thus, to evaluate the selectivity of the proposed sensor to FZD, three structurally related nitro compounds, namely, NTF, NFZ and FTD were prepared at concentration of 10 μM and a DPV analysis was performed. Their structures are provided in Supporting Information (Fig. S1). Both NIP and MIP electrochemical response were compared with the response obtained towards FZD at the same concentration, as expressed in Fig. 8. For each compound, the imprinting factor (IF) was calculated using the following equation: $\text{IF} = i_p(\text{MIP})/i_p(\text{NIP})$, where i_p corresponds to the peak current. The selectivity factor (α) was then calculated as $\alpha = \text{IF}_{\text{FZD}}/\text{IF}_{\text{interferent}}$ and the values are represented in Fig. 8.

The obtained results showed that the current response for FZD after 30 min of incubation on MIP sensor is much higher than those of the other molecules. This fact proves the generation of effective recognition sites complementary to FZD molecule in shape, size, and functional groups, and confirms the success of the imprinting process. Although NTF, NFZ and FTD have a high degree of structural similarity to FZD, the molecular sizes are quite distinct, which led to a considerably less absorption into cavities of MIP-CPE-MWCNTs. The oxidation peaks for NTF, NFZ and FTD corresponds to 35 %, 33 % and 12 % of the FZD peak, respectively. Meanwhile, there is a low significant difference between their intensity of MIP and NIP peak current. Fig. 8 also showed that for NIP-CPE-MWCNTs similar current responses were detected either for FZD or for structural analogues, indicating that no suitable binding sites were produced on the surface of the NIP sensor. Therefore, in the developed MIP-CPE-MWCNTs an excellent recognition selectivity towards FZD molecules becomes obvious.

3.8. Application to water samples

To validate the application of the proposed MIP-CPE-MWCNTs sensor for the analysis of FZD in real samples, tap water and Lis river water collected upstream of WWTP in Leiria were used. Using the standard addition method, samples were spiked with two different concentrations of FZD (0.05 μM and 0.5 μM) and the analysis performed without pre-treatment steps. The obtained recovery values are summarised in Table 4 and were in good agreement with the spiked results. As can be seen, recovery of FZD measured by MIP-CPE-MWCNTs is in the range from 90 to 105 %. This means that the developed sensor has excellent accuracy and is suitable for determining FZD in water samples without the need of laborious pre-treatment steps or methods.

4. Conclusions

For the first time, an efficient and selective MIP electrochemical sensor prepared on CPE were reported for the detection of FZD. QM and MD calculations demonstrated to be a powerful approach in the pre-screening of experimental polymerisation conditions, providing a sustainable strategy towards the design of MIPs. These computational methods avoided the extent and trial-and-error of experimental synthesis. Based on unique properties of MWCNTs, the novel MIP-CPE-MWCNTs greatly enhanced the response signal of FZD and the imprinted cavities formed demonstrated high ability to recognise FZD molecules. Under the optimum conditions, the proposed sensor exhibited a low detection limit of 0.03 μM , as well as sensitive and selective response in the presence of other nitro analogous compounds. The MIP-CPE-MWCNTs was able to detect FZD in spiked tap water and water collected upstream of a WWTP output in the Lis river with appropriate levels of precision, indicating that the developed sensor has potential to be a useful platform to FZD detection in environmental waters.

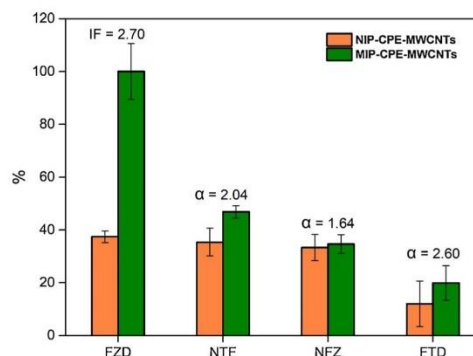


Fig. 8. The oxidation peak current percentage of 10 μM FZD and its analogues on NIP-CPE-MWCNTs sensor and MIP-CPE-MWCNTs.

Table 4

Determination of FZD concentration in spiked water samples with the developed MIP-CPE-MWCNTs sensor^a.

Sample	[FZD] _{add} , μM	[FZD] _{det} , μM	Recovery, %
River water	0		
	0.05	0.052	105
	0.5	0.484	97
Tap water	0		
	0.05	0.051	101
	0.5	0.454	91

^a [FZD]_{add}: FZD concentration added to the sample; [FZD]_{det}: FZD concentration determined in the sample using the MIP sensor developed in this work.

CRedit authorship contribution statement

Patrícia Rebelo: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **João G. Pacheco:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Visualization, Supervision. **Iuliia V. Voroshylova:** Software, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **André Melo:** Software, Data curation, Writing - review & editing. **M. Natália D.S. Cordeiro:** Software, Writing - review & editing, Supervision. **Cristina Delerue-Matos:** Writing - review & editing, Supervision.

Declaration of Competing Interest

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

Acknowledgements

Patrícia Rebelo is grateful to FCT (Fundação para a Ciência e a Tecnologia) for her PhD grant (SFRH/BD/132384/2017). João Pacheco is grateful to FCT for his postdoc grant (SFRH/BPD/101419/2014), financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu and Ministério da Ciência, Tecnologia e Ensino Superior. This work was further supported by UID/UI/50006/2020 with funding from FCT/MCTES through national funds. The authors would like to thank the EU and FCT/UEFISCDI/FORMAS for funding, in the frame of the collaborative international consortium REWATER financed under the ERA-NET Cofund WaterWorks 2015 Call. This ERA-NET is an integral part of the 2016 Joint Activities developed by the Water Challenges for a Changing World Joint Programme Initiative (Water JPI). The authors would like to thank the project Farmasense

(39957) that was funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020) and the Fundo Europeu de Desenvolvimento Regional (FEDER).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.snb.2020.129112>.

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Supporting information

Rational development of molecular imprinted carbon paste electrode for Furazolidone detection: theoretical and experimental approach

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Table S1. Composition of the simulated pre-polymerisation systems.

System	Molar ratio (FZD:AMPS:EGDMA)	Number of molecules						Total number of interaction sites
		FZD	AMPS	EGDMA	AIBN	ACN	DMF	
A	1:1:20	10	10	200	6	2000	-----	18234
B	1:2:20	10	20	200	6	2000	-----	18494
C	1:4:20	10	40	200	6	2000	-----	19014
D	1:6:20	10	60	200	6	2000	-----	19534
E	1:8:20	10	80	200	6	2000	-----	20054
F	1:4:20	10	40	200	6	-----	2000	31014

Table S2. Density (d) and self-diffusion coefficients (D) of the simulated pre-polymerisation systems.

SYSTEM	d, kg m ⁻³	D×10 ⁹ , m ² s ⁻¹					
		FZD	AMP	EGDMA	AIBN	ACN	DMF
A	877.20	0.31 ± 0.00	0.28 ± 0.03	0.50 ± 0.02	0.38 ± 0.07	1.39 ± 0.02	----- -
B	882.50	0.32 ± 0.00	0.29 ± 0.04	0.49 ± 0.01	0.54 ± 0.03	1.42 ± 0.01	----- -
C	893.39	0.34 ± 0.06	0.24 ± 0.05	0.37 ± 0.02	0.40 ± 0.01	1.19 ± 0.01	----- -
D	902.50	0.50 ± 0.06	0.24 ± 0.01	0.34 ± 0.01	0.51 ± 0.02	1.20 ± 0.02	----- -
E	911.96	0.37 ± 0.03	0.18 ± 0.01	0.31 ± 0.02	0.32 ± 0.03	1.02 ± 0.01	----- -
F	1006.67	0.17 ± 0.01	0.16 ± 0.00	0.26 ± 0.00	0.27 ± 0.04	----- -	0.55 ± 0.01

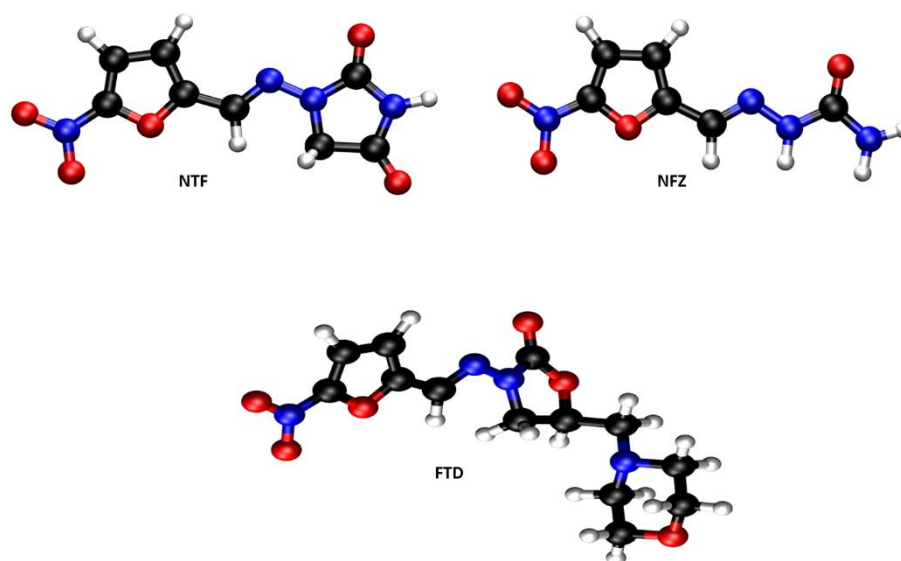
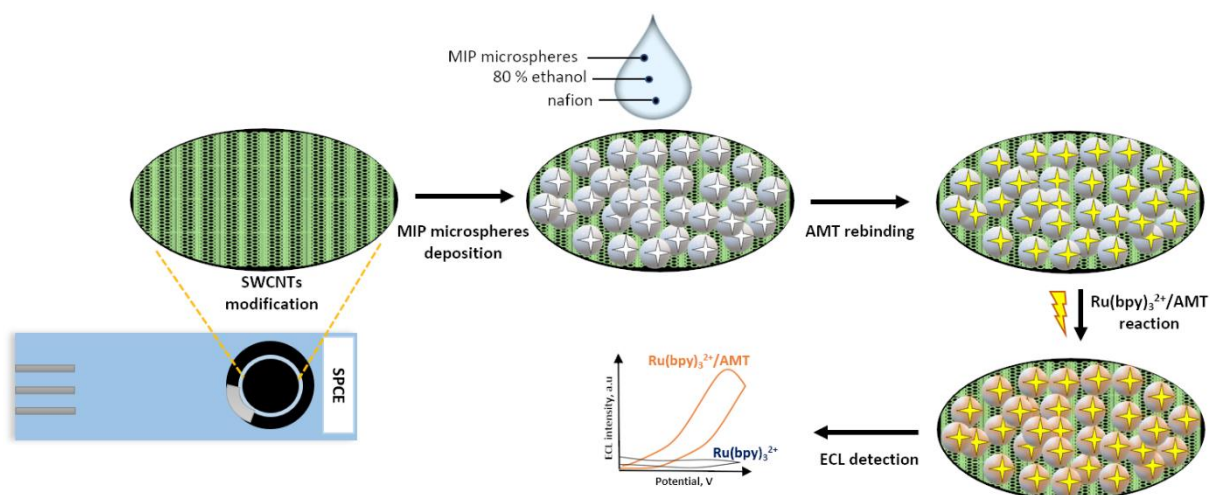


Fig. S1. Chemical structures of NTF, NFZ and FTD used in this study as interferents.

3.3 Development of a molecular imprinted electrochemiluminescence sensor for amitriptyline detection: from MD simulations to experimental implementation

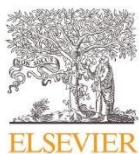
Electrochimica Acta 397 (2021) 139273



Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review, investigation, methodology and writing of the original draft.

Electrochimica Acta 397 (2021) 139273



Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Development of a molecular imprinted electrochemiluminescence sensor for amitriptyline detection: From MD simulations to experimental implementation

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ARTICLE INFO

Article history:

Received 12 July 2021

Revised 31 August 2021

Accepted 15 September 2021

Available online 19 September 2021

Keywords:

Electrochemiluminescence sensor

Molecularly imprinted polymer

Molecular dynamic

Amitriptyline

Water

ABSTRACT

Amitriptyline (AMT) frequent presence in environmental waters reflects the continuous consumption growth and raises issues on the importance of its monitorization. In this work, a sensitive and selective electrochemiluminescence (ECL) sensor was constructed using molecularly imprinted polymer (MIP) recognition element for AMT detection. Molecular dynamics (MD) simulations were performed to select the best functional monomer. Precipitation polymerization was followed to prepare the MIP microspheres using methacrylic acid (MAA) as functional monomer, ethylene glycol methacrylate (EGDMA) as crosslinker and chloroform (CHL) as solvent. The MIP sensor was then prepared on a low cost and disposable screen-printed electrodes (SPCEs), previously modified with single-walled carbon nanotubes (SWCNTs), by drop coating a solution containing the MIP microspheres synthesized. The mechanism of detection was based in the system $\text{Ru}(\text{bpy})_3^{2+}$ /AMT, where AMT acts as co-reactor of $\text{Ru}(\text{bpy})_3^{2+}$ ECL. Several parameters controlling the preparation process of the sensor and AMT detection were optimised. The MIP/SWCNTs/SPCE ECL sensor showed good analytical performance with a linear correlation between ECL signal and the AMT concentration ranging from 0.1 to 200 μM ($R^2 = 0.9991$). The limits of detection (LOD) and quantification (LOQ) were found to be 0.4 μM ($S/N=3$) and 1.5 μM ($S/N = 10$), respectively. The MIP ECL sensor displayed good selectivity to recognise AMT molecules when compared with analogue structures and it was successfully applied in real water samples with good recovery values (90 to 112%). The developed MIP ECL sensor is suitable for integration with portable devices for AMT detection in environmental waters.

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1. Introduction

Amitriptyline (AMT) is one of the oldest tricyclic antidepressant drugs in the pharmaceutical market. It is widely prescribed to treat several central nervous system diseases, such as major depression, anxiety, and bipolar disorders. This psychotropic drug acts mainly by blocking the reuptake of serotonin and norepinephrine to potentiate the activity of these neurotransmitters [1]. Over the last decade, in accordance with an increase antidepressant consumption, AMT has been produced and prescribed in large quantities [2]. Although its benefits to the human health are undoubting, the extensive use of AMT has resulted in its introduction into the en-

vironment. Thus, AMT is commonly detected at measurable concentrations in aquatic ecosystems [3,4]. Since aquatic life is likely to be receiving psychotropic medication with bioaccumulative potential, great concerns about ecotoxicological effects on non-target species have been expressed.

Studies have demonstrated that AMT and its main active metabolite, nortriptyline, have been found everywhere, i.e., in surface water, drinking water, river water, sewages and effluents of wastewater treatment plants (WWTPs) [1,2,5–7]. This is coupled with mounting evidence that, even at low environmental concentrations, AMT can produce significant perturbations on the behavior, reproduction, and normal growth of a wide range of aquatic

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organisms and communities [5,8–10]. In this context, the development of analytical methods to detect and control AMT in environmental waters is of paramount importance.

For quantification of AMT in commercial formulations, biological and water matrices, different methods mostly based on separation and preconcentration techniques have been reported, including high performance liquid chromatography [11–14], gas chromatography [15–17], capillary zone electrophoresis [18,19] and spectrophotometry [20,21]. Despite their numerous advantages, these methods require laborious, costly, and time-consuming procedures, where a sample preparation is frequently a necessary and critical step [1,22]. In contrast, due to their benefits such as simple, fast and sensitive response, electrochemical techniques are becoming an attractive alternative to overcome the limitations found in the aforementioned methodologies. With particular attention to electrochemical sensors, some reports using electrochemical techniques have been proposed for AMT determination [23–26]. However, the ability of sensitively and/or selectively recognize specific target molecules remains a challenge.

Currently, electrochemiluminescence (ECL), which is a special type of chemiluminescence phenomenon, arising from an electrochemical reaction, has received considerable attention in the design of sensors. Taking into account ECL versatility, high sensitivity, broad dynamic range and low background interference, its popularity is of no surprise [27,28]. Furthermore, its combination with molecularly imprinted polymers (MIPs) can develop sensors with the benefits of both high sensitivity and desirable selectivity [29]. In this context, ECL sensors based on MIPs have become one of the most promising approach for the trace target detection in numerous fields, including environmental monitoring [30]. Their application has increased by the introduction of miniaturised devices as screen printed electrodes, which can offer low-cost, portability, sensitive and rapid quantitative information [31,32]. To improve the efficiency of sensing performance, nanomaterials are frequently adopted as enhancers and conducting pathways in typical ECL systems [33–35]. Among them, carbon nanotubes (CNTs) are well-known and established candidates [36,37]. Considering their large specific surface area and conductivity, several works have already demonstrated that the introduction of CNTs can greatly potentiate the electrochemical performance of the MIP sensors [38,39] and increase the ECL intensity [28,40].

MIPs offer remarkable properties like robustness, high specificity towards target molecules, cost-effectiveness and ease of miniaturization [41]. Although they have a generally facile concept, an appropriate choice of monomers with relevant functional groups and efficiency to bind each desirable target compound is typically a quite time-consuming process, often requiring significant amount of chemicals and solvents. Therefore, the development of computational screening methods is particularly important for the rational design and synthesis of MIPs [42]. Despite a limited number of studies reported so far, some MIPs based on results of molecular dynamics (MD) simulations can be found in the literature [43–45]. MD simulations are being successfully used as complementary tool for understanding the prevalent molecular interactions during the imprinting process [46].

In this work, we reported for the first time an ECL sensor based on MIP for AMT detection. After MD simulations, in which a suitable functional monomer was chosen, the MIP were obtained by precipitation polymerization method. Subsequently, the resulting MIP microspheres were immobilized on screen printed carbon electrode (SPCE) surface modified by single-walled carbon nanotubes (SWCNTs). Considering the tertiary amine in AMT structure and its potential to act as co-reactant of tris(2,2'-bipyridyl)ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$), the ECL signal was produced by a sensitive $\text{Ru}(\text{bpy})_3^{2+}$ /AMT reaction. The developed

MIP/SWCNTs/SPCE based ECL sensor was then applied for the selective determination of AMT in spiked water samples.

2. Materials and methods

2.1. Experimental setup

2.1.1. Reagents and sample preparation

Amitriptyline (AMT) hydrochloride (HCl), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA, 98%), 2,2'-azobisisobutyronitrile (AIBN, $\geq 98\%$), single-walled carbon nanotubes (SWCNTs, $\geq 90\%$ carbon basis), tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate ($\text{Ru}(\text{bpy})_3^{2+}$), nafion solution (perfluorinated resin solution 5wt.% in lower aliphatic alcohols and water) and N,N-dimethylformamide (DMF, 99.8 %) were obtained from Sigma-Aldrich. Methanol (MeOH , $\geq 99.9\%$) were purchased from Merck. Acetic glacial acid, ethanol and chloroform (CHL) were supplied by Carlo Erba. For interference studies, imipramine HCl (IMI), doxepin HCl (DOX), clomipramine HCl (CLOM), desipramine HCl (DESI), trimipramine HCl (TRIMI) and nortriptyline HCl (NOR-TRI), purchased from Cerilliant, were used. A stock solution of AMT was prepared daily in phosphate buffer (0.1 M, pH = 7) and used to prepare less concentrated standards. The SWCNTs dispersion was prepared by sonication in DMF for further dilutions in water. Working solutions were freshly prepared and stored in absence of light. Before use, MAA and EGDMA were purified by passing them through a short column filled with neutral alumina (Sigma Aldrich) to remove inhibitors. All the other reagents were used as supplied. Ultra-pure water (resistivity = $18.2 \text{ M}\Omega\cdot\text{cm}$) obtained from a Millipore (Simplicity 185) water purification system was utilised in all experiments. Phosphate buffer solution was prepared with KH_2PO_4 and K_2HPO_4 (Riedel-de-Haën). The water samples were collected from tap, river and public standpipe and were used without pre-treatment.

2.1.2. Instrumentation

ECL experiments were performed by using a portable bipotentiostat/galvanostat combined with a fiber optic, SPELEC (Methrom DropSens, Spain), with controlling software by DropView SPELEC. Screen-printed carbon electrodes (DRP-110, Methrom, Spain) were used as three-electrode system consisted of a 4 mm carbon working electrode, a graphite auxiliary electrode and a silver pseudo electrode. Voltammetric measurements were carried out using a Methrom Autolab PGSTAT 204 potentiostat/galvanostat controlled by NOVA 1.10 software. A Crison pH meter (model microPH 2002) with a combined glass electrode was used for pH measurements. Scanning electron microscope (SEM) images were acquired by FEI Quanta 400 FEG ESEM/EDAX Pegasus X4M equipment at CEMUP (Centro de Materiais da Universidade do Porto).

2.1.3. Synthesis of MIP microspheres and preparation of MIP/SWCNTs/SPCE based ECL sensor

MIP microspheres for AMT detection were synthesised according to the precipitation polymerization method. First, 1 mmol of AMT as template molecule and 4 mmol of MAA as functional monomer were mixed into 50 mL of CHL. Afterwards, 20 mmol of EGDMA and 0.3 mmol of AIBN were added into the above mixed solution, which was purged with nitrogen during 10 min. Subsequently, the mixture was heated at 60°C in a water bath for 4 h. The final reaction precipitates were centrifuged and separated from the reaction medium by filtration. Then, the template was removed through a repeated washing process with a mixture of acetic acid-methanol (1:9, v/v). Finally, the polymer was collected and dried in an oven at 60°C . The non-imprinted polymer (NIP) was also synthesised using the same procedure but without the addition of AMT.

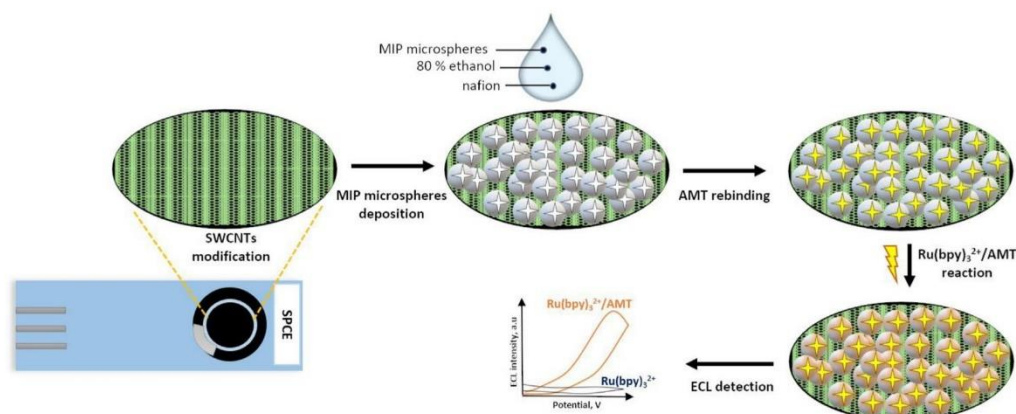


Fig. 1. ECL detection and electrochemical measurements.

The developed polymeric MIP/NIP microspheres were dispersed in 0.05 % nafion and sonicated for approximately 5 min before detection. The preparation process of MIP-SWCNTs modified SPCE is described in Fig. 1. The surface of working electrode was first modified with 5 μL of SWCNTs (0.1 mg mL^{-1}) and the solvent was allowed to evaporate at room temperature. Layers of 2 μL of the MIP/NIP suspension prepared in ethanol (80 % (v/v)) were anchored on the prepared SWCNTs/SPCE and dried to obtain MIP/SWCNTs/SPCE ECL sensor.

2.1.4. ECL detection and electrochemical measurements

In order to realize ELC sensing of AMT, 60 μL containing the desired concentration of the template was dropped on the surface of the modified electrode. After 45 min of incubation, the electrode was rinsed with ultrapure water and dried. Finally, the electrode was put into the ELC cell with 5 mM of $\text{Ru}(\text{bpy})_3^{2+}$ solution to record the response signal. ECL analysis was carried out by cyclic voltammetry (CV) from -0.5 V to $+1.5 \text{ V}$ at 50 mV s^{-1} with a determination time of 45 s. AMT was quantified by calculating the difference, ΔI , between the ECL intensity to the AMT (I_{AMT}) and the background ECL intensity (I_0), ($\Delta I = I_{\text{AMT}} - I_0$).

The electrochemical behavior of differently modified NIP and MIP sensor was also investigated by CV in 0.5 mM of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution. The CV cycles were recorded in the potential range from -0.2 V to 0.5 V with a scan rate of 100 mV s^{-1} .

All experiments were performed at room temperature.

2.2. Computational setup

The MD simulations were carried out as described in our previous work [46] and only the most important details are provided below. All model pre-polymerization mixtures had the same composition: 10 molecules of template, AMT, 40 molecules of functional monomer (acrylic acid (AA), MAA, itaconic acid (IA), 2-vinylpyridine (2VP) or 4-vinylpyridine (4-VP)), 200 molecules of cross-linker, EGDMA, 6 molecules of initiator, AIBN, and 2000 molecules of solvent, CHL. The modelled systems were designated according to the monomer molecule, namely, AMT-AA, AMT-MAA, AMT-IA, AMT-2VP and AMT-4VP. Therefore, the number of interaction sites in the systems varied, in accordance with the monomer molecule size, from 16544 atoms for AMT-AA system to 16784 atoms for the AMT-2VP, AMT-4VP and AMT-IA ones. All molecular species were simulated with the General Amber Force Field (GAFF), generated with the help of ANTECHAMBER module [47].

All MD simulations were carried out in GROMACS 5.1.4 software package [48], following the next protocol: (1) the Packmol package [49] was utilized to randomly place all molecules to initial cubic simulation cells; (2) annealing of each system up to 350 K was employed prior to equilibration stage; (3) the simulations were carried out in the isothermal-isobaric ensemble (NpT) during the equilibration stage and in canonical ensemble (NVT) during the production run, with a time-step of 0.001 ps; (4) the Nose-Hoover thermostat [50,51] (coupling constant: 2.0 ps) was used to maintain the temperature at 298.15 K and, when needed, the Parrinello-Rahman pressure coupling [52] (coupling constant: 8.0 ps) was employed to keep the pressure at 1 bar. The non-bonded Coulombic and Lennard-Jones interactions calculation, as well as neighbour list update, were performed with a 1.2 nm cut-off radius. Particle Mesh Ewald (PME) method [53] was employed to correct the Coulomb interactions beyond the cut-off.

GROMACS inbuilt tools were used to calculate the density, d , by averaging the resulted from the equilibration run energy components, and the self-diffusion coefficient, D , from the mean square displacement of atoms during the production run. Prior to self-diffusion coefficients estimation, the diffusive regime attainment was confirmed by parameter β , as described in our previous works [54–56]. Whenever a physical property was compared with experimental value, the error was estimated as follows: $(|X_{\text{exp}} - X_{\text{sim}}|)/X_{\text{exp}} \times 100\%$, where X is the property obtained experimentally, X_{exp} , or computationally, X_{sim} .

A schematic diagram of the simulated species, showing the most relevant atom types used in this work, is given in Fig. 2a.

3. Results and discussion

3.1. MD simulations

3.1.1. Force Field (FF) validation

Initially, the density, heat of vaporization and self-diffusion coefficient of pure solvent, CHL, were used to test the performance of FF. For this purpose, an additional system, composed only by 2000 solvent molecules, was simulated, according to the established protocol for pre-polymerization mixtures. While d and D were assessed by GROMACS inbuilt tools, as described in Section 2.2, the enthalpies of vaporization, ΔH_{vap} , were calculated according to the formula:

$$\Delta H_{\text{vap}} = U(\text{gas}) - U(\text{liquid}) + RT, \quad (1)$$

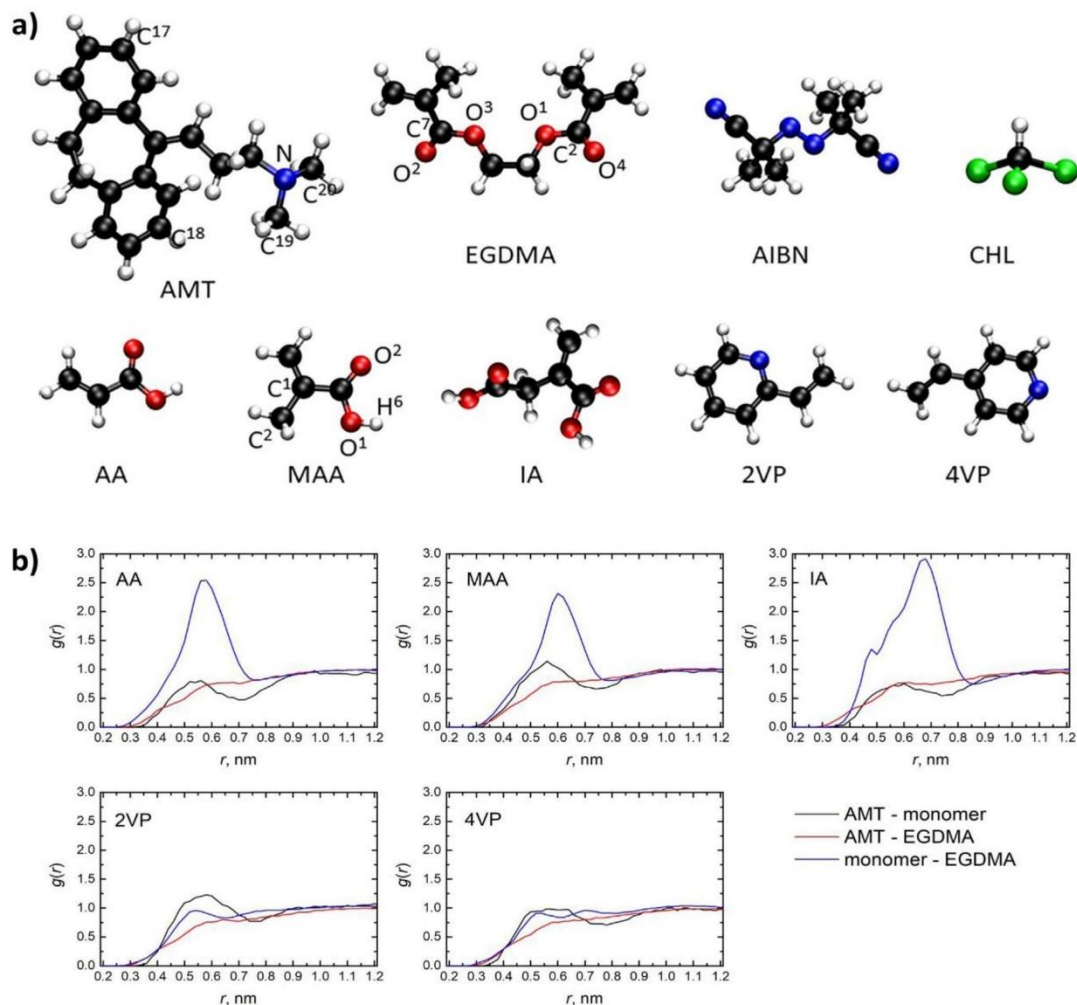


Fig. 2. (a) Geometries of amitriptyline (AMT), acrylic acid (AA), itaconic acid (IA), methacrylic acid (MAA), 2-vinylpyridine (2VP), 4-vinylpyridine (4VP), ethylene glycol dimethacrylate (EGDMA), 2,2'-azobisisobutyronitrile (AIBN) and chloroform (CHL), along with some atom types, used throughout this work. Nitrogen, carbon, hydrogen, oxygen and chlorine atoms are represented by blue, black, white, red and green spheres, respectively. (b) COM-COM radial distribution functions, $g(r)$, between AMT, the monomer and EGDMA molecule in the studied pre-polymerization mixture from the results of MD simulations; the corresponding monomer is indicated on each graph.

where $U(\text{gas})$ and $U(\text{liquid})$ are the total energies in gas and liquids phases, respectively, R is the universal gas constant, and T is the temperature of the simulation. The resulted CHL density ($d = 1451.28 \pm 1.7 \text{ kg m}^{-3}$), self-diffusion ($D = (2.99 \pm 0.1) \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1}$) and heat of vaporization ($\Delta H_{\text{vap}} = 25.76 \pm 0.02 \text{ kJ mol}^{-1}$) are in a good agreement with experimental values (the errors range from 2% for d to 17% for ΔH_{vap}) [57,58]. Thus, the employed FF was found suitable for further study.

3.1.2. Monomer selection

In molecular imprinting processes, functional monomer selection is one of the most significant steps to obtain MIPs with desirable molecular memory. The interactions between the target molecule and the functional monomer should be strong and stable enough to achieve high selectivity and rebinding capacity. In this step, crosslinker properties are also significant to define specifications of the resulting polymer, since crosslinkers fix

functional monomers to templates to produce a polymeric matrix with certain rigidity [59,60]. Thus, to ensure a selection of an ideal functional monomer, radial distribution functions (RDFs) analyzes were performed for predicting the potential interactions existing between a monomer and the target molecule, AMT. At the same time, attention was also given to interactions involving the crosslinker, EGDMA. Based on the typical template-monomer molar ratio of 1:4, five pre-polymerization cells were generated with different potential monomers: AA, MAA, IA, 2VP, 4VP. All systems were prepared with the same crosslinker, initiator and solvent. First the overall microscopic environment of AMT, functional monomers and EGDMA were investigated through calculations of RDFs, $g(r)$. Note that the RDFs for close contacts between AMT, functional monomers and EGDMA, shown in Fig. 2b, were evaluated by centre-of-mass (COM) of all the molecules, with the exception of AMT. After preliminary tests, the AMT interaction site was defined as the COM of its $-N(\text{CH}_3)_2$ group, (See Fig. 2a for ref-

Table 1
Self-diffusion coefficients, D , for the species in the studied systems.

SYSTEM	$D \times 10^9, \text{m}^2 \text{s}^{-1}$ AMT	Functional monomer	EGDMA	AIBN	CHL
AMT-AA	0.52 ± 0.02	0.86 ± 0.07	0.85 ± 0.01	0.93 ± 0.04	1.93 ± 0.04
AMT-MAA	0.77 ± 0.08	0.84 ± 0.01	0.73 ± 0.01	0.82 ± 0.12	1.73 ± 0.01
AMT-IA	0.62 ± 0.01	0.34 ± 0.03	0.63 ± 0.04	0.62 ± 0.01	1.73 ± 0.00
AMT-2VP	0.69 ± 0.03	1.53 ± 0.03	0.81 ± 0.00	0.73 ± 0.01	1.85 ± 0.03
AMT-4VP	0.69 ± 0.01	1.41 ± 0.01	0.76 ± 0.05	0.70 ± 0.09	1.86 ± 0.05

erence), as it was found to be the only part of the molecule with noticeable association activity.

The modelled systems AMT-AA and AMT-MAA (Fig. 2b) showed favourable and similar interactions between the functional monomer (AA or MAA, respectively) and EGDMA, since a defined peak appears at about 0.6 nm, thus confirming the affinity and stability of the complex formed. However, the peak corresponding to close contacts between AMT and functional monomer is more pronounced for the MAA molecule. Closer analysis also revealed that AMT interacts with MAA at a shorter distance when compared to AMT-AA, as it can be seen from the position of RDFs maximum. Therefore, the AMT-MAA system seems to be more promising to establish a strong binding amongst functional monomers and template and, consequently, an efficient imprinting process. Evaluating IA behavior in the system AMT-IA, one can see that although it shows a quite high and defined RDF peak associated to AMT interaction, the distance is longer than that for the other acids. In the case of pyridine-based monomers (2VP and 4VP, in the systems AMT-2VP and AMT-4VP, respectively), only 2VP presents a weak interaction with AMT, while none of them apparently enters in close contact with EGDMA. Thus, as the RDFs analysis suggests, only the AMT-MAA system combines reasonable interactions with both AMT and EGDMA molecules. Accordingly, this system was chosen for further study.

3.1.3. Self-diffusion in the selected pre-polymerization mixture

Theoretical assessment of self-diffusion coefficients, D , is a common measure for the evaluation of the validity of the employed model. Moreover, the estimation of D aids in predicting molecular mobility of the system. Thus, the quality of the simulated AMT-MAA system was also controlled by self-diffusion coefficients for all the present system species in comparison with the D values obtained for the other studied systems. The calculated self-diffusion coefficients are shown in Table 1.

As it can be seen, the self-diffusion coefficients in all the studied systems present reasonable values, comparable to mobility of species in similar systems [46] and thus prove the adequateness of the simulation and of the used models. The highest self-diffusion value of AMT is found in the system AMT-MAA, which is particularly advantageous for template imprinting, since template should be able to diffuse into or out of binding sites within a polymer network [61]. In contrast, highly cross-linked rigid polymer formation is expected to restrict the mobility of the functional monomer and crosslinker in the mixture, meaning stability even after the removal of the template. Additionally, studies have demonstrated that crosslinking density is directly proportional to the binding capacity of the polymers [62]. So, the diffusion of functional monomer and EGDMA within the prepared mixture should be restricted. Comparing the various values obtained for all systems, AMT-MAA system seems to assume this behavior and suggests the suitability of the selected model to the experiments.

3.1.4. Local microstructure in the selected pre-polymerization mixture

Taking into account the above mentioned, local contacts between different atoms were explored to infer how AMT, MAA and

EGDMA interact in the AMT-MAA system. First, specific parts of the molecules were investigated and only the most active sites were selected for the discussion. During RDFs analysis, no relevant interactions involving AIBN or CHL were observed, indicating that these molecules do not disturb the flexibility and mobility of the components of the mixture. The RDFs reported in Fig. 3a and b represent the most relevant close contacts between AMT and MAA, accessed mostly through their carbon atoms. No indication of hydrogen-bonds (H-bonds) formation between AMT and MAA was found, suggesting the prevalence of electrostatic attraction in these interactions. Accordingly, it was found that the $-\text{N}(\text{CH}_3)_2$ group of AMT is the most active site in the aggregation with carbon atoms of MAA (C^1 and C^2), which is consistent with the above analyzed COM RDFs. C^1_{MAA} and C^2_{MAA} have an important role in binding with AMT, but C^2_{MAA} interactions seems to be slightly stronger since their RDFs exhibit more pronounced and defined peaks at shorter distance. In addition, a peak corresponding to $\text{C}^1_{\text{MAA}}-\text{N}_{\text{AMT}}$ binding was also observed, but slightly dislocated towards longer interaction length, indicating that MAA molecule approaches AMT from the side of carbon atoms. In the case of MAA-EGDMA interaction, the sharpness of the first peak observed in Fig. 3c for $\text{H}^6_{\text{MAA}}-\text{O}^{2,4}_{\text{EGDMA}}$ pair at about 0.2 nm revealed the presence of robust H-bonds. The rest of the RDFs observed for MAA-EGDMA close contacts occur at longer distances. Nevertheless, good definition and sharpness of the peaks for $\text{O}^{1/2}_{\text{MAA}}-\text{C}^{2,7}_{\text{EGDMA}}$ and $\text{H}^6_{\text{MAA}}-\text{O}^{1,3}_{\text{EGDMA}}$ curves confirm the stability and strength of MAA-EGDMA interactions. The geometry of typical local arrangements between AMT, MAA and EGDMA molecules, taken directly from the equilibrated simulation cell are shown in the same figure (Fig. 3 d–f). These molecular complexes are consistent with the atom-atom RDFs discussed above. The snapshot of the whole equilibrated box, showed in Fig. S1, portrays the microscopic environment in the studied pre-polymerization mixture.

3.2. Morphological and electrochemical characterisations of MIP/SWCNTs/SPCE

3.2.1. SEM characterisation

To evaluate morphological characteristics of the synthesised imprinted polymer microstructures, SEM analysis was carried out. As it can be seen from the SEM images shown in Fig. 4a and b, after polymerization both NIP and MIP polymers exhibited a porous and spherical structure firmly connected. However, MIP polymer displays obviously larger and more heterogeneous microspheres compared with the NIP, indicating that the presence of AMT in the polymerization mixture influences the growth of the polymer and, consequently, their morphology. The morphology of the SPCEs surface at different preparation stages was also investigated by SEM. Fig. S2 shows typical SEM images of bare electrode (Fig. S2a), SWCNTs/SPCE (Fig. S2b) and SWCNTs-nafion/SPCE (Fig. S2c). It can be observed that the modified SPCEs have different surface morphology when compared with the bare electrode, suggesting that a uniform film with good density has been coated on the electrode surface. It was noted that the nafion solution, used to immobilise the MIP microspheres, has a smooth disposition when coated onto

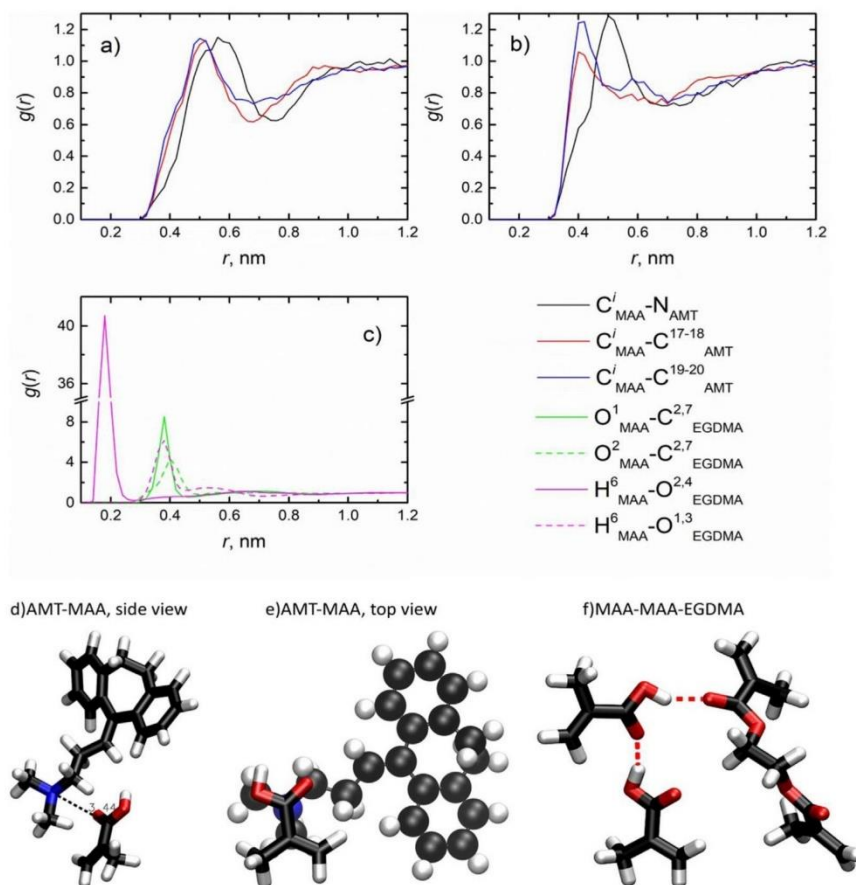


Fig. 3. Atom-atom radial distribution functions, $g(r)$, between specific sites of AMT and MAA molecules (a, b) or MAA and EGDMA molecules (c) in the studied pre-polymerization mixture from the results of MD simulations. C_{MAA}^I corresponds to C_{MAA}^1 (a) and C_{MAA}^2 (b); typical geometry arrangements of AMT, MAA and EGDMA molecules in the pre-polymerization mixture (d-f); red dashed line (f) indicates the H-bond formation, while black dashed line (d) only illustrates the intermolecular distance.

SWCNTs/SPCE. Fig. 4c confirmed the efficient attachment of MIP microspheres on characteristic sponge-like clusters of SWCNTs.

3.2.2. CV step by step sensor construction characterisation

An electrochemical characterisation of the different steps for MIP/SWCNTs/SPCE fabrication was performed by CV technique. Over modifications, the electrochemical response of $[Fe(CN)_6]^{3-/4-}$ probe was used to investigate the electron transfer ability. Fig. 5a shows all the cyclic voltammograms corresponding to the redox reaction of $[Fe(CN)_6]^{3-/4-}$. As shown in this figure, a typical reversible redox process of $[Fe(CN)_6]^{3-/4-}$ was observed at the bare SPCE. After SWCNTs deposition onto SPCE surface, an enhancement of the redox peak current was noted, demonstrating that SWCNTs play an important role in the electron transfer rate. Upon building layers of MIP polymer on the surface of SWCNTs/SPCE, a significant reduction in the current response can be seen. This indicated a successful immobilisation of a compact film, thus hindering the charge transfer of the redox probe. When the MIP/SWCNTs/SPCE was incubated in 10 μM AMT solution, the current response was re-suppressed. This can be attributed to the rebinding of AMT molecules on the imprinted cavities, blocking the infiltration of $[Fe(CN)_6]^{3-/4-}$ into electrode.

3.3. ECL behavior of AMT

The ECL signal of the $Ru(bpy)_3^{2+}/AMT$ was investigated in 5 mM $Ru(bpy)_3^{2+}$ prepared in phosphate buffered solution (0.1 M, pH=8) with a scan range from -0.5 V to +1.5 V at 500 $mV s^{-1}$. The signal was recorded after incubation in AMT solution for 45 min. Different electrode modifications were studied and the results are expressed in Fig. 5b. At bare SPCE no significant ECL signal was observed, which can be explained by the very weak adsorption of AMT molecules at the carbon ink. After modification with SWCNTs, an enhancement of the ECL signal was registered, suggesting an increase of electrode's surface area, as well as electrical conductivity, which contributed to aid fast electron transference. Although there has been some unspecific adsorption of AMT molecules at SWCNTs/SPCE surface, the binding had no significant effect without polymer microspheres.

At NIP/SPCE there was slightly increase in the signal, which can be attributed to the ability of the polymer microspheres to adsorb AMT molecules. Nevertheless, the signal of MIP/SPCE was much higher than NIP/SPCE. These results shown a higher adsorbing of AMT at MIP microspheres, indicating the successful formation of cavities that selectively bind to AMT. The modification with SWC-

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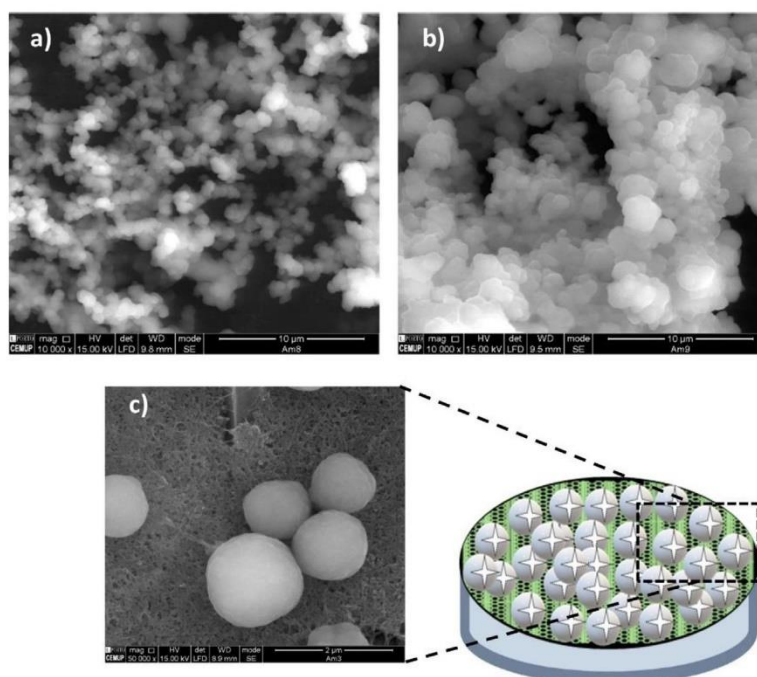
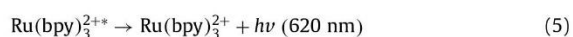
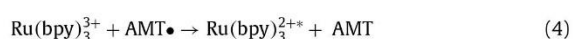


Fig. 4. Morphology surface SEM images of NIP (a) and MIP microspheres (b) obtained after polymerization and subsequent immobilisation onto SPCE surface (c) covered with SWCNTs.

NTs before polymer deposition, both in NIP/SPCE and MIP/SPCE shown an increase in the ECL signal. These results demonstrate that SWCNTs were essential to improve the methods sensitivity.

The ECL mechanism of $\text{Ru}(\text{bpy})_3^{2+}$ /AMT is similar to the common $\text{Ru}(\text{bpy})_3^{2+}$ /tripropylamine (TPA) system. In our work, AMT molecules selectively adsorb to MIP/SWCNTs/SPCE sensor act as co-reactant of (instead of TPA) $\text{Ru}(\text{bpy})_3^{2+}$ to produce a ECL signal via application of a positive potential. The proposed mechanism is expressed below:



Initially $\text{Ru}(\text{bpy})_3^{2+}$ and AMT are oxidised to $\text{Ru}(\text{bpy})_3^{3+}$ and AMT radical cation ($\text{AMT}^{\bullet+}$), respectively. Then, the short-life radical cation loses a proton and produces $\text{AMT}^{\bullet}$, which will react with $\text{Ru}(\text{bpy})_3^{3+}$ in an electron transfer process to generate the excited state of $\text{Ru}(\text{bpy})_3^{2+*}$. Finally, the later will eradicate light emission at the wavelength of 620 nm.

3.4. Optimisation of the MIP/SWCNT/SPCE preparation and experimental conditions

To obtain good ECL signal of AMT, various critical parameters, responsible for controlling the performance of the developed

sensor, were optimised including (a) concentration of MIP microspheres; (b) number of deposited MIP layers onto SPCE surface, (c) concentration of $\text{Ru}(\text{bpy})_3^{2+}$ and (d) incubation time. The measurements were performed in triplicate and the results are demonstrated in Fig. 6.

During the preparation of MIP/SWCNTs/SPCE, the content of deposited MIP microspheres ranging from 1 mg L^{-1} to 8 mg L^{-1} was evaluated (Fig. 6a). As expected, with the increasing amount of MIP microparticles, a significant enhancement in the ECL intensity of AMT in $\text{Ru}(\text{bpy})_3^{2+}$ system was registered until a maximum of 6 mg L^{-1} . This result is reasonable because more MIP microspheres on the SPCE surface provide more recognition sites and, consequently, more AMT molecules were adsorbed. However, when the amount of MIP microspheres was higher than 6 mg L^{-1} , the ECL signal decreased, which may be attributed to SPCE conductivity decreasing in the presence of excessive amount of MIP microspheres on its surface [63]. So, the optimum value of 6 mg L^{-1} for concentration of MIP microspheres in the deposition solution was identified and used in the following experiments.

Then, keeping constant the parameter mentioned above, the number of MIP layers coated on the working electrode surface was investigated (Fig. 6b). The best ECL intensity was obtained when the thickness of the deposited MIP film was composed by three layers of $2 \mu\text{L}$ of MIP solution. With the number of layers less or more than three, ECL intensity decreased. Although layer-by-layer assembly represents a favorable strategy to produce strong ECL signal, dense MIP layers seem to work as a barrier against the ECL process, resulting in weak ECL response. Thus, three layers of $2 \mu\text{L}$ of MIP solution were adopted and we conclude that 0.05% (w/w) of nafion is enough to achieve the maximum binding without undesirable effects in the stability and sensitivity of MIP/SWCNTs/SPCE sensor.

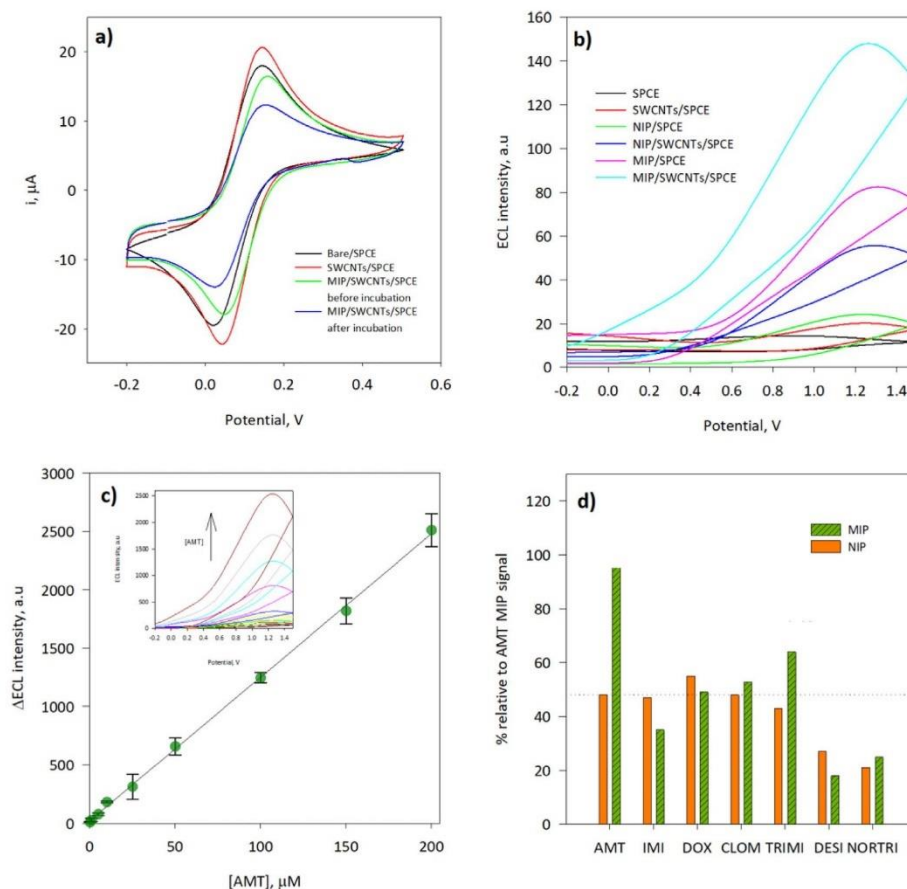


Fig. 5. (a) Cyclic voltammograms of 0.5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ used to characterize the electrochemical behavior of MIP/SWCNTs/SPCE after different modifications. (b) ECL responses of different electrodes in 5 mM $\text{Ru}(\text{bpy})_3^{2+}$ in phosphate buffered solution (0.1 M, pH= 8) after 45 min of incubation in AMT solution; (c) The calibration curve of AMT determination and the corresponding ECL signals obtained in the range 0.1–200 μM in the inset; (d) ECL response of MIP/SWCNTs/SPCE and NIP/SWCNTs/SPCE to AMT and other six closer compounds.

The ECL signal strongly depends on both the ECL emitter and the co-reactant. So, the concentration of $\text{Ru}(\text{bpy})_3^{2+}$ and AMT incubation time were indispensable parameters considered for ECL measurement. Fig. 6c depicts the concentration of $\text{Ru}(\text{bpy})_3^{2+}$ in the range of 5–15 mM. As can be seen, the ECL intensity slightly increased from 5 mM to 8 mM, while higher concentrations of $\text{Ru}(\text{bpy})_3^{2+}$ lead to decline of ECL efficiency. This condition can be related to the positive and negative effect caused by the molar ratio between ECL emitter and co-reactant. Higher $\text{Ru}(\text{bpy})_3^{2+}$ concentrations cause a higher demand of co-reactant, which overwhelmed the positive effect of increased $\text{Ru}(\text{bpy})_3^{2+}$ content. Additionally, taking into account that the difference between 5 mM and 8 mM of $\text{Ru}(\text{bpy})_3^{2+}$ was not significant to justify the use of large amounts of this emitter, 5 mM was chosen as the optimal concentration to $\text{Ru}(\text{bpy})_3^{2+}$ for subsequent experiments.

Similarly, the effect of AMT incubation time was studied (Fig. 6d). For various time periods (4, 8, 20, 30, 45, 60 min), the ECL responses of MIP/SWCNTs/SPCE were recorded and a gradual increase in the ECL intensity was observed when the incubation time was extended, meaning more AMT molecules were rebinding in the imprinted cavities over time. It reached a maximum intensity after 45 min of incubation. Contrarily, when the time incubation

was increased to 60 min, the ECL intensity decreased, suggesting an electrostatic hindrance arising from the more compact AMT molecules adsorbed. Therefore 45 min was selected as an appropriate incubation time.

Finally, as the introduction of SWCNTs was beneficial to sensitivity of MIP/SWCNTs/SPCE sensor, the experiment result indicated that 5 μL of a solution containing 0.1 mg mL⁻¹ of SWCNTs was optimal.

3.5. Analytical performance

Based on the optimisation of experimental conditions described above, the linear relationship between the ECL intensity ($\Delta I = I_{\text{AMT}} - I_0$) and the AMT concentration was investigated. As it can be observed in Fig. 5c, the ECL signal gradually increases with the AMT concentration in the tested range between 0.1 and 200 μM. Good linear relationship was obtained and the regression equation ($n=9$) was: $\Delta I_{\text{ECL}} = 12.29 \times [\text{AMT}] (\mu\text{M}) + 22.40$, with correlation coefficient of 0.9991.

The limits of detection and quantification were estimated to be 0.4 μM ($S/N = 3$) and 1.5 μM ($S/N = 10$), respectively.

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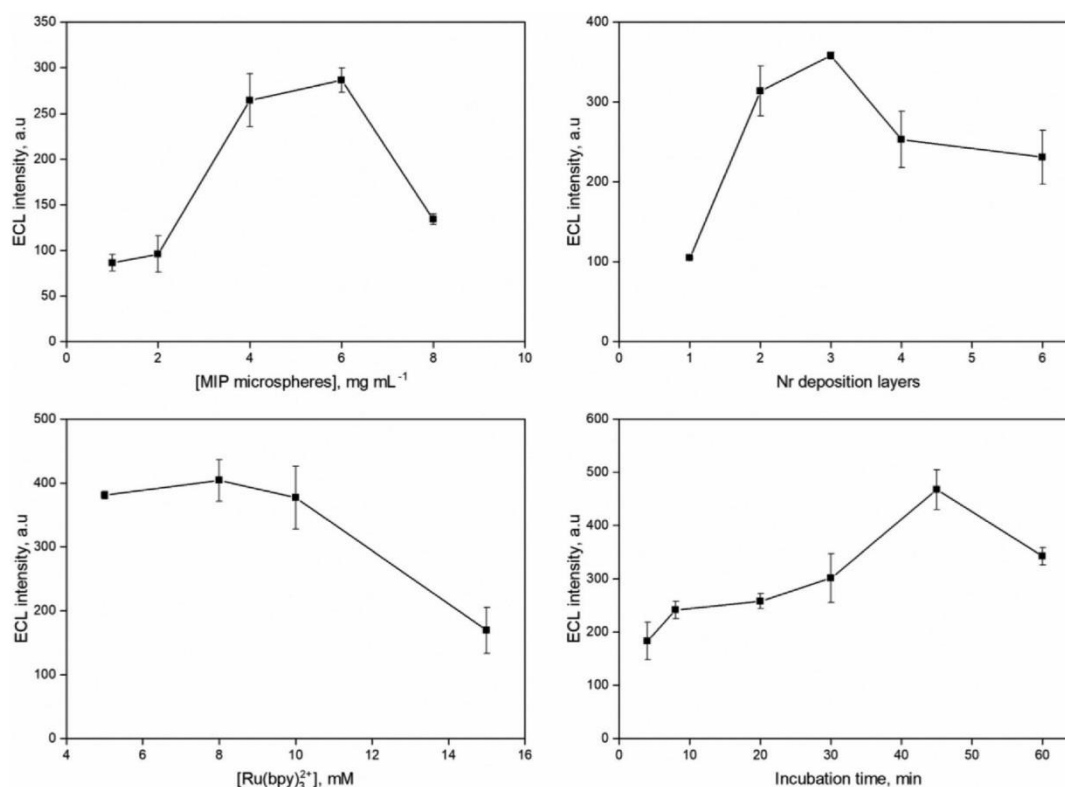


Fig. 6. (a) Variation of ECL signal with the concentration of MIPs microspheres in the deposition solution; (b) Variation of ECL signal with the number of deposition layers; (c) Variation of ECL signal with the concentration of Ru(bpy)₃²⁺; (d) variation of ECL signal with the incubation time.

In reproducibility measurements, four different electrodes prepared with the same composition were tested on 10 μ M AMT concentration. The obtained relative standard deviation (RSD) of 3.9 % demonstrated a good precision and stable response of the developed MIP sensor.

3.6. Selectivity studies

One of the major MIPs characteristics is the ability to selectively recognise the target molecule. Therefore, the selectivity of the proposed MIP sensor to AMT was evaluated. Six structurally related compounds, namely, IMI, DOX, CLOM, TRIMI, DESI and NORTRI were selected. Both NIP/SWCNTs/SPCE and MIP/SWCNTs/SPCE ECL responses to AMT were registered, under the optimised conditions, and compared with the response obtained towards the selected compounds at the same concentration, as expressed in Fig. 5d. Results were presented in percentage of ECL signal of AMT at MIP/SWCNTs/SPCE. For each compound, the imprinting factor (IF) was calculated using the following equation: $IF = \text{ECL signal (MIP)} / \text{ECL signal (NIP)}$, where ECL signal corresponds to $\Delta I (I - I_0)$.

After the analysis was completed, it was clearly observed that the ECL signal of AMT on the MIP/SWCNTs/SPCE electrode was higher than those of other compounds. Therefore, we may conclude that selective binding sites complementary to AMT molecules in shape, size and functional groups were created and the developed MIP sensor has higher sensitivity towards AMT among all tested similar structures.

Among the other molecules tested, the higher signal was registered for TRIMI with a signal slightly higher than NIP/SWCNTs/SPCE. For all the other compounds the signals were similar or lower the NIP/SWCNTs/SPCE signal of AMT. At the same time, the MIP and NIP signals of each molecules showed no significative differences, indicating that these signals can be related with the non-specific binding. Noteworthy, the ECL signal can only be registered if a co-reactor of Ru(bpy)₃²⁺ is present during the measurement. This fact improves the sensor's selective response, due to a limited number of existent compounds that can act as co-reactor.

3.8. Application to water samples

To test the application of the developed method for real sample analysis, the constructed MIP/SWCNTs/SPCE ECL sensor was applied for the determination of AMT in three different water samples, namely, river water, tap water and spring water. Samples were spiked with two different concentrations of AMT (5 μ M and 50 μ M) and the analysis was performed without any pre-treatment procedure. Using the standard addition method, the recoveries were calculated and summarised in Table 2. The results shown that the developed MIP/SWCNTs/SPCE ECL sensor has good accuracy and is suitable for AMT determination, with recoveries in the range between 90 and 112 %. The sensor also proved to be suitable for detection of AMT in complex samples without the need of laborious and expensive pre-treatment steps.

Table 2
Determination of AMT concentration in spiked water samples with the proposed MIP/SWCNTs/SPCE sensor^a.

Sample	[AMP] _{add} , μM	[AMP] _{det} , μM	Recovery, %
River water	0		
	5	5.23	104.6
	50	45.13	90.3
Tap water	0		
	5	5.58	111.7
	50	48.43	96.8
Spring water	0		
	5	5.16	103.2
	50	49.82	99.6

^a [AMT]_{add}: AMT concentration added to the sample; [AMT]_{det}: AMT concentration determined in the sample using the MIP sensor developed in this work

4. Conclusions

A ECL sensor for AMT selective and sensitive determination was constructed for the first time based on a modified disposable and low cost SPCE electrode. The electrode was modified with SWCNTs, that enhanced the ECL signal and improved the sensitivity of the method. Selectivity was achieved by using MIPs with specific binding sites to AMT as selective recognition element. MD simulations supported the MIP preparation, firstly allowing to choose the most appropriate functional monomer for the pre-polymerization solution and then providing a molecular-level interpretation of interactions in the mixture. Under the optimised conditions, the proposed sensor was highly sensitive to AMT molecules when compared with other analogues chemical structures. The ECL signal is based on Ru(bpy)₃²⁺/AMT (co-reactor) system, improving the selectivity because few drugs' molecules can act as co-reactor and produce an ECL signal. The prepared MIP/SWCNTs/SPCE ECL sensor has good analytical performance and was successfully applied to the determination of AMT in spiked real water samples. When compared with common chromatographic methods it has several advantages, such as portability, simplicity, low-cost, lower time of analysis and solvent economization.

Declaration of Competing Interest

None

Credit authorship contribution statement

Patrícia Rebelo: Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **João G. Pacheco:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. **Iuliia V. Voroshylova:** Software, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **M. Natália D.S. Cordeiro:** Software, Writing – review & editing, Supervision. **Cristina Delerue-Matos:** Writing – review & editing, Supervision.

Aknowledgments

Patrícia Rebelo (SFRH/BD/132384/2017) and João Pacheco (SFRH/BPD/101419/2014) are grateful to Fundação para a Ciência e a Tecnologia (FCT) and European Union (EU) for their grants, financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu (FSE) and Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). The work was supported by the project Farmasense (39957) that was funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020)

and the Fundo Europeu de Desenvolvimento Regional (FEDER). This work was further supported by UID/QUI/50006/2020 (LAQV-REQUIMTE) with funding from FCT/MCTES through national funds. The authors would like to thank the EU and FCT/UEFISCDI/FORMAS for funding, in the frame of the collaborative international consortium REWATER financed under the ERA-NET Cofund WaterWorks 2015 Call. This ERA-NET is an integral part of the 2016 Joint Activities developed by the Water Challenges for a Changing World Joint Programme Initiative (Water JPI).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.electacta.2021.139273.

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Supporting Information

Development of a molecular imprinted electrochemiluminescence sensor for amitriptyline detection: from MD simulations to experimental implementation

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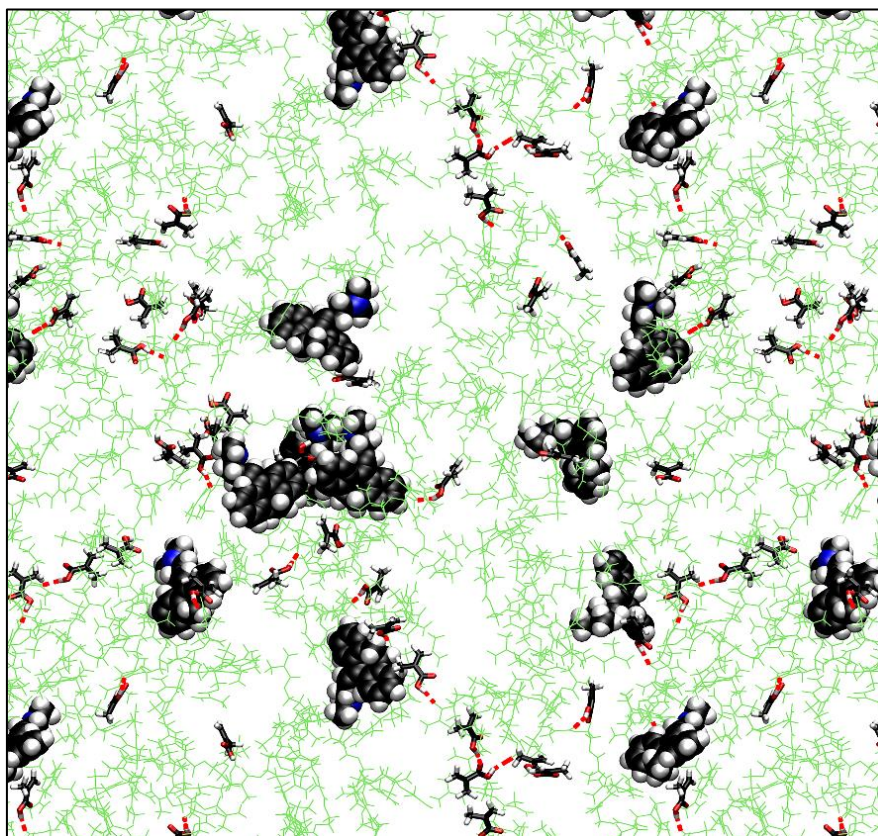


Fig. S1. Snapshot of the equilibrated pre-polymerization mixture from MD simulations. The AMT, MAA and EGDMA molecules are presented as colored van der Waals spheres, colored licorice and green wireframe structures, respectively. Carbon, nitrogen, oxygen, and hydrogen atoms of AMT and MAA molecules are represented as black, blue, red and white spheres, respectively. The rest of the molecules (AIBN and CHL) are omitted for clarity. Red dashed lines represent the existing in the mixture H-bonds.

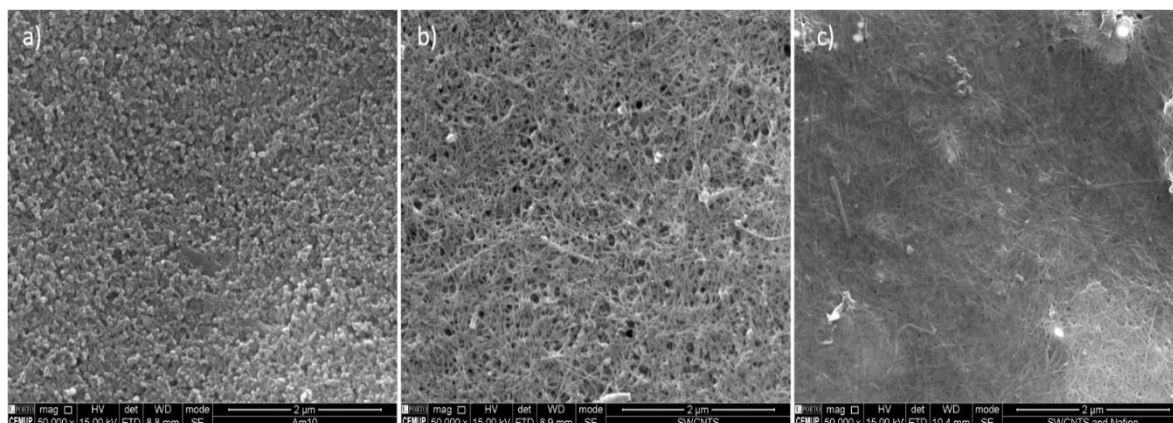
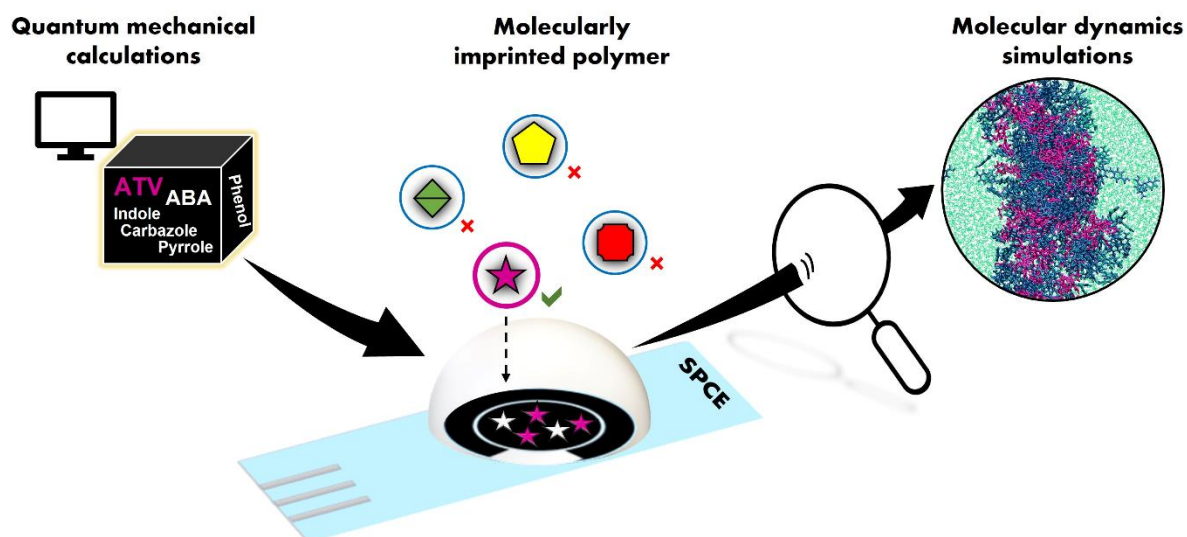


Fig. S2. SEM images of (a) bare SPCE, (b) SWCNTs/SPCE and (c) SWCNTs-Nafion/SPCE.

3.4 A simple electrochemical detection of atorvastatin based on disposable screen-printed carbon electrodes modified by molecularly imprinted polymer: experiment and simulation

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Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review, investigation, methodology and writing of the original draft.

Highlights

- A disposable electrochemical MIP sensor for atorvastatin (ATV) detection was designed.
- Efficient prediction of a functional monomer was achieved based on QM calculations.
- MD simulations allowed to understand the ATV monomer interaction.
- High selectivity and sensitivity were obtained by a direct detection method.
- The sensor was successfully applied in water samples.

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A simple electrochemical detection of atorvastatin based on disposable screen-printed carbon electrodes modified by molecularly imprinted polymer: Experiment and simulation



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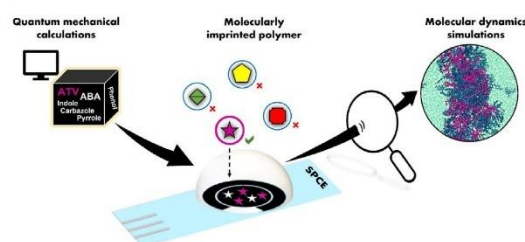
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HIGHLIGHTS

- A disposable electrochemical MIP sensor for atorvastatin (ATV) detection was designed.
- Efficient prediction of a functional monomer was achieved based on QM calculations.
- MD simulations allowed to understand the ATV-monomer interaction.
- High selectivity and sensitivity were obtained by a direct detection method.
- The sensor was successfully applied in water samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 20 August 2021
Received in revised form
10 November 2021
Accepted 27 December 2021
Available online 29 December 2021

Keywords:

Statins
Atorvastatin
Electropolymerisation
Electrochemical sensor
Molecularly imprinted polymer
Environmental analysis

ABSTRACT

Atorvastatin (ATV) is a statin member consumed in high quantities worldwide. In response to that, the occurrence of ATV in environmental waters has become a reality, highlighting the need of rapid and sensitive analytical devices for its monitoring. In this work, the first electrochemical molecularly imprinted polymer (MIP) sensor for the detection of ATV in water samples is presented. Computational studies were conducted based on quantum mechanical (QM) calculations and molecular dynamics (MD) simulations for rational selection of a suitable functional monomer and to study in detail the template-monomer interaction, respectively. The sensor was prepared by electropolymerisation of the selected 4-aminobenzoic acid (ABA) monomer with ATV, acting as template, on screen printed carbon electrode (SPCE). Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques were applied to characterise the modified electrode surfaces. The quantitative measurements were carried out with differential pulse voltammetry (DPV) in 0.1 M phosphate buffer (pH = 7). After investigation and optimisation of important experimental parameters, a linear working range down to 0.05 $\mu\text{mol L}^{-1}$ was determined with a correlation coefficient of 0.9996 and a limit of detection (LOD) as low as 0.049 $\mu\text{mol L}^{-1}$ (S/N = 3). High sensitivity and selectivity of the prepared sensor were demonstrated with the ability to recognise ATV molecules over its closer structural analogues. Moreover, the sensor was

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<https://doi.org/10.1016/j.aca.2021.339410>

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quickly and successfully applied in spiked water samples, proving its potential for future on-site monitoring of ATV in environmental waters.

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1. Introduction

In the last decades, the most sensitive Earth's ecosystems have been significantly modified by anthropogenic activities [1]. Regarding natural aquatic systems, questions about their quality are continually being discussed mainly due to the widespread detection of pharmaceutical compounds in different aquatic compartments [2,3]. Although these compounds are generally found at trace environmental levels, their acute and chronic ecotoxicological effects on wild biota have been reviewed and reported by many researchers [4–7]. Of the wide range of pharmaceutical compounds classes encountered into environmental waters, statins are now among the most frequently detected groups [8].

Statins, also known as cholesterol-reducing agents, are increasingly prescribed for preventing and treating cardiovascular diseases that are the number one cause of global mortality [9]. Accordingly, they belong to the top-selling human pharmaceuticals in European countries and United States [8,10], among which atorvastatin (ATV) stands out as the most popular statin member [11,12]. Considering that ATV has been intensively used, it is not surprising that this compound is ubiquitous in aquatic environments around the world. Available literature studies have reported detectable levels of ATV in wastewater treatment plant (WWTP) influents and final effluents [13–17]. Recently, the presence of ATV in Brazilian drinking water treatment plants was noted by Reis et al. [18]. Environmental ATV concentrations have been also documented in surface waters receiving discharges from WWTPs [1,19–21]. These assessments reflect the general problem associated with the efficiency of wastewater treatment processes in removal and degradation of pharmaceuticals [22]. Consequently, several species may be continuously exposed to ATV levels with potential to disturb their normal growth and reproduction [5,8,23,24]. Therefore, it is very important to build sensing systems for determining ATV in environmental waters.

For environmental monitoring, there is a growing need for analytical methods and technologies enabling specific, simple, selective and rapid responses. In this context, molecularly imprinted polymers (MIPs), used as a recognition element for electrochemical sensors development, have become an interesting class of materials with promising ability to quantify pharmaceuticals in aqueous matrices [25]. While electrochemical methods offer such advantages as rapid analysis, operation simplicity and low cost, MIPs provide high binding affinity and selectivity for the target molecule desired [26,27]. Furthermore, MIPs have mechanical and chemical stability in a wide range of experimental conditions and solvents, taking advantages over their biological counterparts [28]. Sensing systems based on this combination have showed good analytical performance and thus, they have attracted more and more attention in the recent years [25]. For the integration of MIPs in electrochemical sensor platforms, different strategies can be applied, including electropolymerisation, sol-gel process and photopolymerisation [29,30]. Among these methods, electropolymerisation has unique superiorities related with the direct production of MIPs on precise location of the electrode surface [31]. In a very simple and rapid way, the thickness, homogeneity and the density of the polymer layer can be well controlled under different electrodeposition conditions [32,33]. Many researchers have

successfully employed electropolymerisation for MIPs preparation on a variety of conductive electrodes. However, because of increasing request for on-spot monitoring, screen printed electrodes (SPEs) have gaining noteworthy enhancements in environmental analysis [34,35]. SPEs are miniaturised and disposable electrochemical sensors, which satisfy the need for reproducibility, cost-effectiveness, sensitivity and portability *in situ* monitoring [36,37]. Additionally, in agreement with green chemistry concepts, their operation requires only small sample volumes, reducing the use of unfriendly environment solvents and waste production. Over the past few years, in order to prevent the synthesis of MIPs by trial and error, computational studies have been increasingly explored as useful screening methods capable of handling the complexity of molecular imprinting process [34,38–40]. Quantum mechanical (QM) calculations and molecular dynamics (MD) simulations have been reported for the prediction of functional monomer-target compound interactions, as well as for exploring and analysing the molecular imprinting recognition mechanism [41–43].

Currently, some studies based on the electrochemical detection of ATV in pharmaceutical formulation and biological matrices are available in the literature [44–47]. However, to the best of our knowledge, no electrochemical sensor based on MIP to detect ATV in water has been developed. Analysis of ATV in environmental samples currently relies on the use of chromatographic techniques [14,19,48] that are relatively slow and require expensive instrumentation, skilled operator and laborious pre-treatment of the samples [45,46].

In the present research, we report for the first time a simple, cost-effective and highly selective MIP integrated with SPE for electrochemical detection of ATV in water samples. Herein, preliminary search of suitable functional monomer was carried out by QM calculations. The MIP layer was prepared by electropolymerisation of the selected functional monomer on the surface of SPE and, after optimising experimental parameters, the accuracy of the system composition was investigated by MD simulations. The proposed method proved to be promising and the new electrochemical sensor was successfully applied for determination of ATV in spiked water samples.

2. Materials and methods

2.1. Reagents and solutions

Atorvastatin calcium salt trihydrate (ATV), 4-aminobenzoic acid (ABA), N, N-dimethylformamide anhydrous (DMF, ≥ 99.8%), analogues of ATV (pravastatin sodium salt hydrate (PRV), simvastatin (SMV), rosuvastatin calcium (RSV), fluvastatin sodium hydrate (FLV), lovastatin (LOV) and pitavastatin (PTV)) and potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihydrate were obtained from Sigma-Aldrich. Ethanol and hydrochloric acid (HCl) were acquired from Carlo Erba. All chemicals employed were of analytical grade and were used without further purification.

Stock solutions of ATV (1 mmol L⁻¹) were prepared in ethanol-H₂O (1:1, v/v) mixture and stored in a freezer below 8 °C. Various concentrations of ATV were prepared daily by appropriate dilution of the stock solutions in phosphate buffer (0.1 M, pH = 7). Ultra-pure water used in all experiments (resistivity = 18.2 MΩ cm)

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was obtained from a Millipore (Simplicity 185) water purification system. The water samples were collected from tap, spring and Asprela river in Porto and were used without any pre-treatment.

2.2. Instruments

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using a Metrohm Autolab PGSTAT 204 potentiostat/galvanostat coupled to a computer using a NOVA 1.10 software for data acquisition. Electrochemical impedance spectroscopy (EIS) was recorded out by a Metrohm Autolab PGSTAT 128 N potentiostat/galvanostat controlled by NOVA 1.6 software. SPCEs used in this work were purchased from Metrohm (DRP-110) and consist of a three-electrode configuration: a circular carbon working electrode with the diameter of 4 mm, a silver pseudo-reference electrode and a carbon counter electrode. pH measurements were carried out by a Crison pH meter (model micropH 92002) with a combined glass electrode. All experiments were conducted at room temperature (25 °C).

2.3. Computational approach

2.3.1. Theoretical search of the functional monomer

In order to screen and select the functional monomer capable of forming stable complexes with ATV, a virtual library of ten electropolymerisable monomers, including 4-ABA, indole, 4-aminothiophenol, carbazole, mercaptobenzimidazole, pyrrole, aniline, phenol, o-phenylenediamine, and 1-vinylimidazole were created. First, the chemical structure of all the molecules was built with the aid of Visual Molecular Dynamics (VMD) software [49]. Gaussian'09.RevD program package [50] was then used to assess the pre-optimisation of their geometric configurations at a PM6 or a PDDG semi-empirical level. AutoDock program [51] within the VMD plugin VsLab [52] was applied to construct the template-monomer model through the combination of ATV with different functional monomers. Finally, the conformational optimisations and stabilisation energies for the complexes in vacuum were obtained by density functional theory (DFT) at M06-2X/6-31 + G* level.

The binding energies differences (ΔE) between ATV and different functional monomers were estimated according to equation (1):

$$\Delta E = E(\text{complex}) - E(\text{template}) - E(\text{monomer}), \quad (1)$$

where $E(\text{complex})$ is the formation energy of template-monomer complex, $E(\text{template})$ and $E(\text{monomer})$ denote the energy of template and functional monomer, respectively.

2.3.2. MD simulations

MD studies were performed similarly to our previous work [42]. All simulations were carried out with the GROMACS 5.1.4 software package [53]. Due to the complexity of the target molecule, ATV, it was decided to thoroughly explore all the inter-particle interactions in a single system composition, rather than explore the influence of compounds' concentration. The investigated pre-polymerisation mixture represented the experimentally used template-to-monomer ratio, i.e., 1:8. Although the synthesis of this MIP occurs in phosphate buffer (see Section 2.3), for computational study the molecules were placed in water to mimic the solvent. Thus, the simulation cell comprised 50 ATV molecules, 400 ABA molecules and 10 000 molecules of water, resulting this way in quite a large cell of 40 600 atoms. The General Amber Force Field (GAFF) was used for all molecular species as received from module ANTECHAMBER [54]. Regarding water, TIP3P FF [55] was employed

taking into account its adequateness for simulations of pharmaceuticals, as proved by a number of studies [56,57]. The initial simulation box was generated with Packmol code [58], that allowed to place all the necessary molecules randomly in the simulation box.

The simulation methodology included the following steps: (i) energy minimisation with maximum force tolerance of $10 \text{ kJ mol}^{-1} \text{ nm}^{-1}$; (ii) pre-equilibration for 100 ps in NVT ensemble at temperature as low as 100 K to allow for initial relaxation and resolving of possible packing problems; (iii) further relaxation in another short pre-equilibration run of 500 ps at 298.15 K in NPT ensemble; (iv) annealing for 1 ns in NVT ensemble with heating of the system up to 370 K, to overcome the rest of possibly existing energy barriers; (v) equilibration for 1 ns (in NPT); and finally (vi) production run of 20 ns (in NVT). For production and equilibration stages the bonds between heavy atoms and hydrogens were constrained. All simulations were computed under pressure of 1 bar. The rest of the details (temperature and pressure coupling methods, applied cut-offs, electrostatic interaction corrections beyond the cut-off, algorithms for equations of motion integration and bonds constraining, etc.) can be consulted in great detail from our previous work [59].

The in-built GROMACS tools were used for the calculations of radial distribution functions (RDFs) and analysis of hydrogen-bonding (H-bond) interactions. The GRACE software was used for the numerical integration of RDFs for coordination numbers (CNs) assessment. Geometry of the simulated species, with the atom numbering used throughout this work, is given in Fig. 1.

2.4. Preparation of the optimised MIP sensor and electrochemical analysis

For preparation of MIP-modified SPCE, a homogeneous and thin MIP film was obtained by electropolymerisation of phosphate buffer mixture containing the functional monomer, 0.8 mmol L^{-1} ABA and template, 0.1 mmol L^{-1} ATV, on the surface of the SPCE. The electro-polymerisation process was performed using CV between potential range of -0.2 V and 1.4 V at a scan rate of 100 mV s^{-1} for consecutive 20 cycles. After this step, ATV molecules extraction was carried out with phosphate buffer and DMF- H_2O (1:9) solution during 10 min and 5 min, respectively. Next, the electrodes were carefully washed with deionised water and dried at room temperature. To evaluate the efficiency of MIP sensor, the same procedure was employed to prepare non-imprinted polymer (NIP sensor), but in the absence of ATV during electropolymerisation.

The recognition of the template molecules was then made by incubation of one drop ($40 \mu\text{L}$) of different concentrations of ATV for 30 min onto the prepared modified SPCEs. The electrodes were washed to eliminate any faintly bound ATV, and its rebinding response were directly evaluated by DPV measurements in phosphate buffer over a potential range from 0.3 V to 1.2 V , pulse amplitude and scan rate of 50 mV s^{-1} .

To characterise the MIP films, the surface modification steps were additionally investigated by CV and EIS experiments by measuring the current and resistance variations of 5 mmol L^{-1} $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in phosphate buffer dropped on the SPCE surface. CV characterisation analyses were operated cycling the potential from -0.2 V to 0.5 V with a scan rate of 100 mV s^{-1} . EIS spectra was recorded at the potential of 0.2 V , frequency range from 0.1 to 10 Hz with an amplitude of 10 mV and under a number of frequencies distributed logarithmically of 60. Experimental EIS data was fitted using the Randles equivalent electrical circuit.

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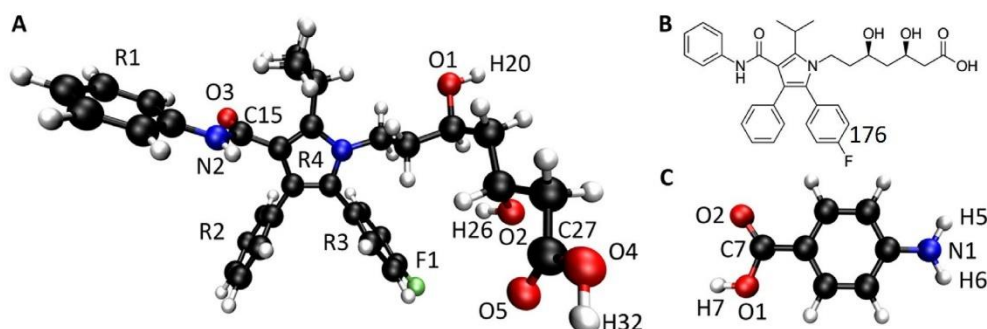


Fig. 1. Optimised geometries of ATV (A) and ABA (C) molecules and schematic representation of ATV molecule (B) with atom types, used throughout this work. R1, R2, R3 and R4 indicate the ATV molecule rings. Nitrogen, carbon, oxygen, hydrogen, and fluorine atoms are represented by blue, black, red, white, and green spheres, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results and discussion

3.1. Rational selection of the functional monomer

One of the most important stages in design and synthesis of high affinity MIPs for the respective analyte is the selection of an adequate functional monomer. However, this task can take an extensive experimental work where large material quantities are involved. Thus, a number of QM calculations were undertaken to estimate the potential association energies of molecular complexes and, consequently, make a rational choice of a functional monomer that will produce an ATV MIP with improved molecular memory. The systems were built at 1:1 (ATV: functional monomer) molar ratio and a DFT approach was used to optimise the geometry conformation of the possible complexes. The binding energies between ATV and each tested functional monomer were calculated through Eq (1); the results are listed in Table S1. In theory, the most suitable monomer for preparation of desired MIP should have the highest stability and hence the highest absolute interaction energy of the template-monomer complex [60]. As observed, the highest binding score belongs to ATV-ABA complex ($-76.064 \text{ kJ mol}^{-1}$), which is $1.705 \text{ kJ mol}^{-1}$ more stable than the following most stable complex (ATV-Indole, with $-74.359 \text{ kJ mol}^{-1}$) and is $39.850 \text{ kJ mol}^{-1}$ more stable than the least energetically favoured combination studied in this work (ATV-1-vinylimidazole, with $-36.214 \text{ kJ mol}^{-1}$). Therefore, it can be inferred that the non-covalent interactions established between ABA and ATV are stronger than in the other complexes studied. Thus, ABA was subsequently selected as the functional monomer to prepare the MIP sensor with high recognition capacity to ATV.

3.2. Electrochemical deposition and behaviour of ATV on SPCE surface

After selecting ABA as the most suitable functional monomer, the molecular imprinting of ATV molecules for the synthesis of MIP layer was performed via electropolymerisation directly on SPCE surface. Under the conditions described above, this was carried out by continuous 20 CV cycles of 0.1 mmol L^{-1} ATV and 0.8 mmol L^{-1} ABA in a 0.1 M phosphate buffer solution at a scan rate of 100 mV s^{-1} .

For comparison, Fig. 2A and its inset shows the resulting CV curves obtained with ATV (MIP after polymerisation) or without ATV (NIP) in the electropolymerisation medium, respectively. As seen from the figures, in the potential range from -0.2 to 1.4 V ,

significant differences can be observed in the first scan of MIP and NIP polymeric films. Initially, both displayed a cathodic peak at about -0.55 V that can be attributed to the electrochemical oxidation of the ABA at the SPCE surface. However, in the preparation of MIP (Fig. 2A), two well-defined peaks were recorded at 0.7 V and 0.8 V , while they did not appear in the NIP film electro-generation. As expected, this finding reveals the oxidation of the embedded ATV molecules that are becoming part of the polymeric chain. During the electrodeposition of the polymer, ATV molecules diffuse towards the working electrode and produce a far higher number of recognition sites than those are generated in the NIP film, this being the key process in the molecular imprinting. Also, in the case of MIP and NIP synthesis, the current intensity gradually decreased from the 1st to 20th scan, thus confirming the formation of an imprinted compact polymeric film which suppressed the voltametric response.

Following electropolymerisation, preliminary DPVs experiments were performed in phosphate buffer to check the incorporation of ATV in the MIP film, as well as its removal and subsequent rebinding. Fig. 2B displays the DPVs signals recorded between $+0.3$ and $+1.2 \text{ V}$ on NIP and MIP SPCE freshly electropolymerised. As observed, their electrochemical behaviour is quite different. Only MIP SPCE exhibited an oxidation peak at around 0.6 – 0.7 V , which proves the successful entrapment of ATV molecules inside the MIP layer. Further, consideration was given to different extraction solutions with ability to remove these molecules and, consequently, reveal imprinted cavities that will be the specific recognition sites to ATV during rebinding step. For this purpose, it was found that the exposition of the prepared MIP sensor to phosphate buffer and DMF, allowed an efficient release of ATV without destroying the polymeric material stability. This is supported by DPV analysis, where no current peak was observed (Fig. 2B). When the electrodes were incubated in phosphate buffer solution containing ATV molecules, the electrochemical response of the MIP sensor was much higher than that of the corresponding NIP. Despite the ability of ABA to bind ATV molecules, the minimal response observed to the NIP sensor after incubation represents a non-specific binding. Therefore, the great difference between the intensity of the peak current value of the MIP and NIP proved the successful construction of MIP layer with ATV cavities, which in turn allowed sensor to absorb more target analyte molecules.

3.3. Optimisation of the MIP fabrication conditions

To improve the performance of the designed MIP sensor,

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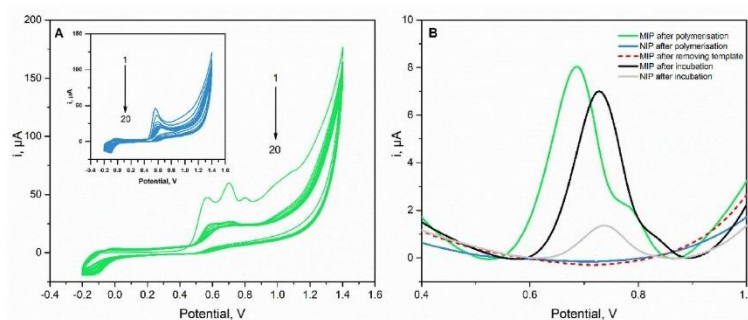


Fig. 2. (A) Cyclic voltammograms for the electropolymerisation of 0.8 mmol L⁻¹ ABA and 0.1 mmol L⁻¹ ATV (MIP sensor) in 0.1 M phosphate buffer onto SPCE surface. The inset figure shows the electrochemical polymerisation of 0.8 mmol L⁻¹ ABA in the absence of ATV (NIP sensor). (B) DPV results of MIP and NIP sensor after different modifications.

experimental parameters including the monomer (ABA) and template (ATV) concentrations, the number of electro-polymerisation scan cycles, the incubation time and the pH of incubation solution were investigated and optimised. During optimisation process, the influence of each factor on electrochemical response was studied in NIP and MIP sensor by DPV and the results are presented in Fig. 3.

3.3.1. Effect of the polymerisation mixture components concentration

Since the formation of template cavities and binding properties of the MIP is established by the affinity of the template molecule and functional monomer, optimising their concentrations in the

pre-assembly solution is of great importance. In our study, to determine the effect of monomer concentration on the response of both NIP and MIP sensor, ABA concentration was varied from 0.2 mmol L⁻¹ to 3 mmol L⁻¹, while the concentration of ATV was kept constant. As it is shown in Fig. 3A, the response of NIP and MIP sensor to ATV increases with increasing ABA concentration, suggesting that higher amount of monomer is favourable to create imprinted sites on the polymeric matrix. On the other hand, to improve the sensitivity of ATV detection, the difference between the peak current intensity of NIP and MIP sensor should be as high as possible [61]. And, a decrease in this difference was observed at very high ABA concentrations, which could be ascribed to the presence of irregularly shaped cavities. As maximum sensitivity

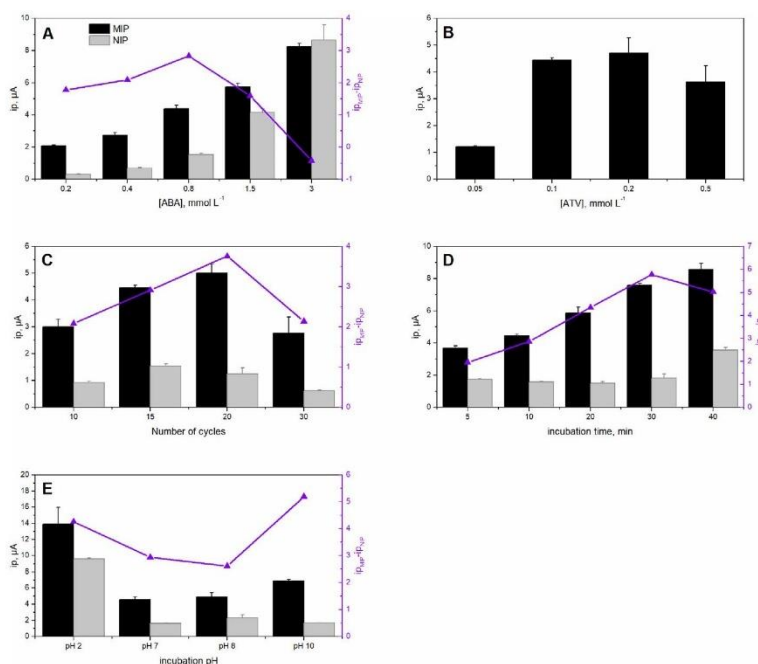


Fig. 3. Optimisation of experimental parameters for determination of ATV at concentration of 10 μmol L⁻¹. Variation of the peak current intensity with the concentration of (A) ABA and (B) ATV in electropolymerisation solution; (C) number of polymerisation cycles; (D) incubation time; (E) incubation pH value. The error bars indicate the standard deviation of three replicate determinations.

should be achieved without compromising selectivity, the optimum ABA concentration of 0.8 mmol L^{-1} was chosen for further experiments. Similarly, the effect of the ATV concentration during the film electrodeposition was evaluated in the range of $0.05\text{--}0.5 \text{ mmol L}^{-1}$. The results presented in Fig. 3B show that the current response of the MIP sensor initially increased with the increase of the ATV concentration up to 0.1 mmol L^{-1} . When the concentration was higher than 0.2 mmol L^{-1} , there was a decrease in the intensity, probably because of a reduction in the conductivity of the MIP layer caused by agglomerations and not bonded ATV molecules. Taking into account that the electro-oxidation current presents insignificant change between 0.1 mmol L^{-1} and 0.2 mmol L^{-1} ATV concentrations, 0.1 mmol L^{-1} was selected for all recognition measurements. This final concentration of mixture components translates in a template to monomer ratio of 1:8.

3.3.2. Effect of number electropolymerisation cycles

After optimisation of monomer and template concentration, the thickness and permeability of the polymeric film was controlled by modifying the number of cycles applied during the electropolymerisation process (Fig. 3C). Under variations of 10, 15, 20 and 30 scan cycles, a considerable decrease in the analytical performance of the MIP sensor was found below and above 20 cycles. Although extensive cycles favour the generation of more imprinted sites, they also result in thicker sensing film with poor site accessibility [28,62]. So, if the produced film is too thick, the diffusion of ATV molecules can be negatively affected with proportional influence on rebinding ability and reproducibility of the MIP sensor. In addition, the highest difference between the electrochemical response of NIP and MIP sensor towards ATV was also obtained by applying 20 scan cycles in the polymerisation. Therefore, 20 cycles were used to produce an optimum film thickness in the proposed MIP sensor.

3.3.3. Effect of incubation time

To study the relationship between the sensor current response and the rebinding time of ATV, NIP and MIP sensors were left in contact with $40 \mu\text{L}$ of ATV solution for different periods (5, 10, 20, 30 and 40 min). As it is clear from Fig. 3D, there is a gradual enhancement in the peak current intensity of the MIP sensor with time, indicating that the bonding capacity of the ATV molecules with the surface of the modified SPCE is improved with time. On the contrary, no significant effect was observed in the electrochemical response of the NIP sensor until 30 min exposition. As expected, this result is consistent with the absence of specific binding sites to ATV molecules on the NIP sensor surface. When incubation time exceed 30 min, the current response of NIP sensor increased as a result of higher contributions from non-specific interactions. Consequently, an incubation time of 30 min was adopted in the followed sensing applications.

3.3.4. Effect of incubation solution pH

In another incubation study, the influence of pH on DPV response of the proposed sensor was evaluated. After incubation with solutions at different pH values such as HCl (0.1 M) and phosphate buffer with pH varied from 7 to 10, it was verified that pH has a strong impact on the sensitivity and selectivity of the MIP sensor towards ATV molecules (Fig. 3E). Despite of MIP sensor achieving the maximum of sensitivity at acidic values, the imprinting effect is disturbed. Interactions between ATV and the imprinted film seem to be facilitated at alkaline solutions. Based on the results, the optimal pH value for incubation was set at 10 because it provided sensitivity adjoined with the highest selectivity.

3.4. Investigating the nature of imprinting by MD simulation

To gain a better insight into the possible interactions, occurring between ATV and ABA upon a binding event within the experimentally used polymerisation mixture, MD simulations were carried out. To closely reproduce the experimental conditions and achieve a more accurate physical description, water was added as a solvent. At the same time, it was assumed that no water molecules were interfering with interactions between ATV and ABA. Thus, as a preparative step for conducting MD study, a simulation box was built, ensuring random insertion of molecules, as depicted in Fig. S1A. As it can be seen from the figure, the ATV, ABA and water molecules, represented in purple, blue and green structures, respectively, were uniformly distributed at this stage. The next snapshot, shown in Fig. S1B, portrays the simulation box after performing all MD simulations procedures, described in Section 2.3.2. As it can be observed, after the MD study the system acquired a totally different arrangement with ATV molecules concentrated at the centre of the box and surrounded by ABA molecules. This result demonstrates the strong affinity between ATV and ABA without influence of solvent effect, highlighting its ability to achieve the desired degree of rebinding and selectivity.

3.4.1. Local structure and H-bonds

Radial distribution functions, $g(r)$, (RDFs) were calculated for a qualitative evaluation of the types of intermolecular interactions occurring in the pre-polymerisation system between ATV and ABA molecules. After preliminary tests, the most active atoms of the studied molecules were identified and only these sites were considered for this discussion. The addressed sites are labelled in Fig. 1. As expected, $g(r)$ functions reported in Fig. 4 revealed a significant degree of interaction between ATV and ABA, which is essential for the successful imprinting. As follows from the below-discussed, the observed binding affinities between ATV and functional monomer result mainly from hydrogen bond formation. Indeed, closer examination showed that ATV has a good affinity for the template by the two oxygen atoms of the carboxyl group (O^5 and O^4 , see Fig. 1) and the respective hydrogen (H^{32}), the two hydroxyl oxygen (O^1 and O^2) and hydrogen atoms (H^{20} and H^{26}) of the side chain, followed by the oxygen of the carbonyl group (O^3). At the same time, interactions of ABA are dominated by oxygen (O^2 , see Fig. 1) and hydrogen (H^7) of the carboxylic group, as well as by the two hydrogen atoms of the amine group (H^5 and H^6). The interaction observed between the carboxyl groups of the ATV and ABA active sites seems to be the most favourable binding, with high and defined peaks corresponding to $\text{O}^5_{\text{ATV}}\text{--H}^7_{\text{ABA}}$ and $\text{H}^{32}_{\text{ATV}}\text{--O}^2_{\text{ABA}}$ close contacts at about 0.2 nm , supporting the idea of H-bonds existence in the pre-polymerisation mixture. When these bindings occur, the contribution of O^4_{ATV} atoms at longer distance can be also observed, which suggests that their conformational position are comparatively less favourable than O^5_{ATV} to establish strong and defined interactions. In other words, the carboxyl oxygen of ATV molecules is preferred by the carboxyl hydrogen of ABA over hydroxyl oxygen of ATV for establishing of intermolecular contacts. When considering the hydroxyl groups of ATV, a similar behaviour was presented by O^2_{ATV} , O^1_{ATV} , $\text{H}^{26}_{\text{ATV}}$ and $\text{H}^{20}_{\text{ATV}}$ during complexation with ABA. In fact, while $\text{O}^{2,1}_{\text{ATV}}\text{--H}^7_{\text{ABA}}$ interactions remain the featured point of close molecular contacts, the interactions of $\text{O}^{2,1}_{\text{ATV}}$ with $\text{H}^{5,6}_{\text{ABA}}$ also appear to acquire a significant contribute to ATV-ABA affinity. On the contrary, for the interaction between carbonyl group oxygen of ATV and ABA, a higher peak was observed for $\text{O}^3_{\text{ATV}}\text{--H}^{5,6}_{\text{ABA}}$ contact and not for $\text{O}^3_{\text{ATV}}\text{--H}^7_{\text{ABA}}$. This is the only interaction site where ABA molecule was found to turn mostly its amine group towards the ATV molecule. The rest of the shown in Fig. 4 $g(r)$ curves represent contacts

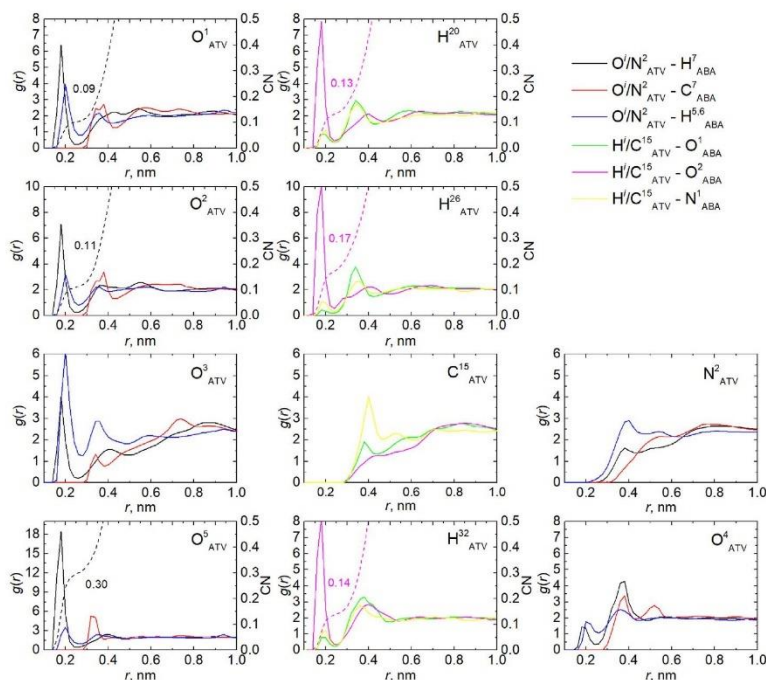


Fig. 4. Radial distribution functions, $g(r)$, (left axis, solid lines) showing probabilities of interaction between specific sites of ATV and ABA molecules and, where applicable, corresponding coordination numbers, CNs, (right axis, dash lines) in the studied pre-polymerisation system from the results of molecular dynamics simulations. Numbers represent CNs according to colour code in the legend. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

between atoms, directly attached to principal active sites, i.e., N^1_{ABA} , C^{15}_{ATV} , N^2_{ATV} atoms. These RDFs help in the interpretation of the monomer molecules position with the respect to the template molecule.

Although hydrogen bonding plays a significant role on the ATV-ABA binding, other moderate noncovalent interactions are formed. As suggest the RDFs calculated between the geometric centres of the rings of ATV and ABA molecules, the aromatic ring of ABA is capable of forming π - π stacking interactions with the four ring-structures of the template. As can be seen in Fig. 5, the contribution of π - π stacking interactions established between rings is influenced by the geometry of ATV molecule. Due to its position, the ATV pyrrole ring is the most susceptible to form favourable interactions with the benzene ring of ABA.

Considering the RDF results, a quantitative evaluation of coordination numbers (CNs) of the most active ATV interaction sites by ABA molecules were accessed, as well as characterisation of H-bonds forming in the mixture were performed. These analyses were carried out for the most pronounced peaks in the RDFs, i.e., $O^{5,2,1}_{ATV} - H^7_{4-ABA}$, $H^{32,26}_{ATV} - O^2_{4-ABA}$ and $H^{20}_{ATV} - O^1_{4-ABA}$. To obtain the CNs, the RDFs were integrated up to the first minimum on the corresponding interaction. The corresponding CNs are reported in the graphs (Fig. 4) and show a good agreement with the strength of each interaction. Indeed, as it can be seen, the highest CN, 0.30, is obtained for the most pronounced close contact, $O^5_{ATV} - H^7_{ABA}$.

Finally, an investigation of the extent and stability of all H-bonding network was carried out following the framework of Luzar and Chandler geometrical criteria [63]. According to these criteria, the distance between the oxygens of two different molecules should be less than 3.5 Å and simultaneously the angle formed by

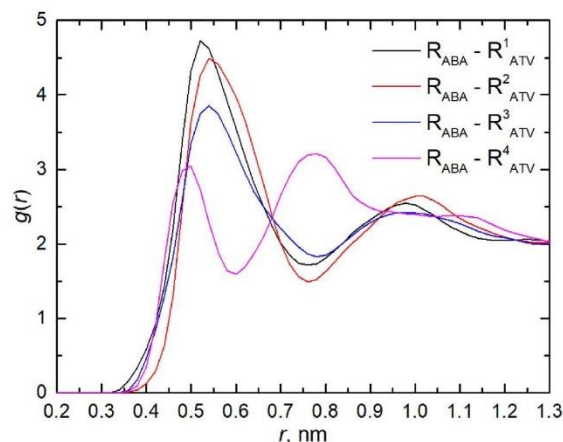


Fig. 5. Radial distribution functions, $g(r)$, showing probabilities of interaction between aromatic rings of ATV and benzene ring of ABA molecules. For rings designations see Fig. 1.

$O-H \cdots O$ should be less than 30° (here $\cdots$ denotes H-bond). Table 1 contains the occurrence and lifetime of the H-bonds obtained for the studied system. The results reinforce the facts presented previously from RDFs analysis. Contacts with more pronounced RDF peaks have a higher average number of hydrogen bonds per frame. As it is clearly seen, the most numerous and stable (i.e., presenting

Table 1
Average number of hydrogen bonds per frame and their lifetimes (in picoseconds in parentheses).

ABA atoms/ATV atoms	H ⁷	H ^{5,6}	O ¹	O ²	Total
O ¹	4.84 (18.4)	9.17 (4.98)	—	—	14.01
O ²	5.27 (21.5)	7.52 (4.25)	—	—	12.79
O ³	3.24 (9.90)	14.7 (4.18)	—	—	17.94
O ⁴	1.65 (5.27)	4.92 (2.23)	—	—	6.57
O ⁵	14.2 (21.5)	8.47 (3.44)	—	—	22.67
H ²⁰	—	—	1.11 (3.52)	6.30 (15.5)	7.41
H ²⁶	—	—	0.47 (2.9)	8.22 (17.4)	8.69
H ³²	—	—	0.94 (4.7)	6.64 (15.1)	7.58

the longest average lifetime) H-bonding interaction between ATV and ABA species is provided by O⁵_{ATV} - H⁷_{ABA}, highlighting its importance in the imprinting quality. At the same time, the ATV interaction with ABA's amino-group via H^{5,6}_{ATV}-O³_{ABA} sites can be also pointed out due to the number of H-bonds per frame, being, however, five times less durable than the most prominent one. From these results, it can be concluded that hydrogen bonding is the dominant driving force within the pre-polymerisation mixture, showing that MD simulations may reveal the competing interactions during the growing process of the polymer.

3.5. Electrochemical characterisation of modified electrodes

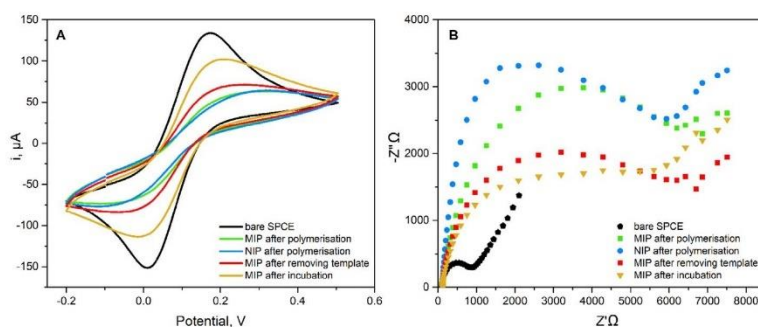
The stepwise construction of the modified SPCE was electrochemically monitored through CV and EIS experiments using 5 mmol L⁻¹ [Fe(CN)₆]^{3-/4-} as redox probe. The results obtained with the bare SPCE and different modified electrodes are shown in Fig. 6. As demonstrated in Fig. 6A, the bare SPCE displays a typical pair of reversible redox peaks for [Fe(CN)₆]^{3-/4-}. After electropolymerisation, a remarkable decrease in the oxidation/reduction peak current of the mediator was registered for both NIP and MIP sensors. This behaviour is consistent with the formation of a non-conductive polymer, hindering the electron conduction at the SPCE surface. No significant differences in the peak current intensities of NIP and MIP were recorded. By removing ATV molecules from the MIP, the permeability of the polymeric matrix increased because of the unoccupied cavities. As a result, the electron exchange also increased, clearly enhancing the peak current of the redox probe. Interestingly, template rebinding led, once again, to an increase of the redox activity at MIP SPCE. This may be explained based on improvements of the electron transfer by conductive bridges formed during molecular recognition.

Similar behaviours were observed by EIS, where the additional information of such surface modifications was acquired. The impedance spectrum presented in Fig. 6B shows the charge-

transfer resistance (R_{ct}) offered by the modified films, which is usually measured from the diameter of the Nyquist plot semicircle at higher frequencies. The Nyquist plots for both NIP and MIP showed the successful deposition of the polymer film. Compared with the bare SPCE, the R_{ct} visibly increased in both cases after electropolymerisation, which are in good accordance with the CV results. However, here it was possible to observe differences between the impedance behaviour of NIP and MIP film. For NIP, the surface was sensibly more resistant to electron transfer than MIP surface, possibly due to the absence of ATV molecules that influence the properties of the final polymer. Owing to the removal of the ATV from the MIP film, a significant decrease in the R_{ct} was remarked, since cavities formed are free again and there are more available access for [Fe(CN)₆]^{3-/4-} through the MIP layer. Finally, with the reloaded of ATV in the specific sites of the MIP sensor, the resistance decreased again, indicating that ATV molecules interaction promotes the occurrence of the electrochemical reaction, as previously stated in the CV analysis. In general, these results agree with the results of CV and seem to confirm the success of the imprinting concept.

3.6. Analytical performance and stability of the MIP sensor

Under the optimum experimental conditions, the sensing performance of the fabricated sensor was verified upon incubations of ATV at various concentrations (Fig. 7). The sensor response was directly measured by DPV and an increase in the oxidation current from low to high concentrations was registered. Indeed, the relationship between the oxidation peak intensity and the concentration exhibited an excellent linear dependence in the range of 0.05 $\mu\text{mol L}^{-1}$ to 2 $\mu\text{mol L}^{-1}$ ($r = 0.996$, $n = 3$). The limits of detection (LOD) and quantification (LOQ) were calculated to be 0.049 $\mu\text{mol L}^{-1}$ and 0.162 $\mu\text{mol L}^{-1}$, respectively, using the following equations: $\text{LOD} = 3 s/m$ and $\text{LOQ} = 10 s/m$, where s is the standard deviation of the intercept and m is the slope of the

**Fig. 6.** Characterisation of the stepwise construction of the sensor by (A) CV and (B) EIS in 0.1 M phosphate buffer containing 5 mmol L⁻¹ [Fe(CN)₆]^{3-/4-}.

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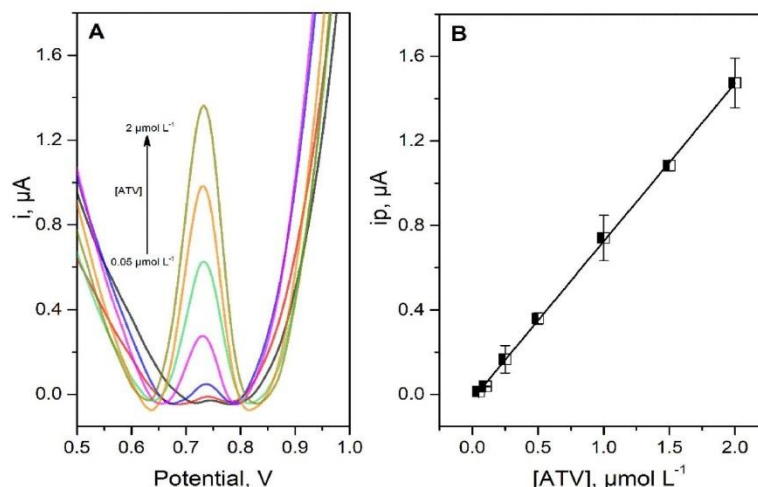


Fig. 7. (A) DPV responses obtained from MIP sensor after incubation in different concentrations of ATV and (B) the correspondent linear relationship between the peak current intensity measured and the concentration of ATV. The error bars indicate the standard deviation of three replicate determinations.

calibration plot. In reproducibility measurements, four MIP sensors were prepared independently by the same procedure and their responses to $1.5 \mu\text{mol L}^{-1}$ ATV were analysed. The response peaks intensity showed a relative standard deviation (RSD) of 6.1% that confirms the reproducibility of the proposed sensor.

3.7. Selectivity studies

An important aspect of molecularly imprinted sensor operation is its selectivity towards the target even in the presence of other similar or interfering molecules. Thus, to assess the selectivity of the proposed sensor, potential interference from six statins with close structural similarities to ATV, namely, FLV, LOV, SMV, PRV, RSV and PTV, were examined. Their corresponding DPV signals for both NIP and MIP sensors were compared with the signal obtained to ATV at the same concentration ($10 \mu\text{M}$), as expressed in Fig. 8.

From the results, it can be easily noted that these interfering

species do not have significant effect on the performance of the developed sensor. The MIP sensor exhibited a much greater recognition for ATV molecules than that for other tested analogues. Moreover, sensors responded only to FLV, LOV and SMV, while no electrochemical responses were recorded for PRV and RSV. This high specific binding ability for detection of ATV proves the generation of memory cavities complementary to the pharmaceutical in shape, size and functional groups, and again confirms the success of the imprinting process. The current response measured to FLV, LOV and SMV corresponds to 49%, 32% and 3% of the ATV response, respectively, being more comparable to non-specific signals showed by them in relation to NIP sensor. Accordingly, the proposed sensor has an obvious selectivity to ATV molecules.

3.8. Analyte detection in water samples

To evaluate practical applicability of the proposed sensor in detecting ATV using real water samples, recovery experiments were carried out by spiking tap water, spring water and river water samples with known concentrations of ATV ($0.1 \mu\text{M}$ and $0.5 \mu\text{M}$). The obtained values, summarised in Table 2, show good agreement with the spiked results. Recovery values, ranging between 90% and 104% were achieved, validating the accuracy of the MIP sensor and its practical suitability to detect ATV in water samples without any pre-treatment.

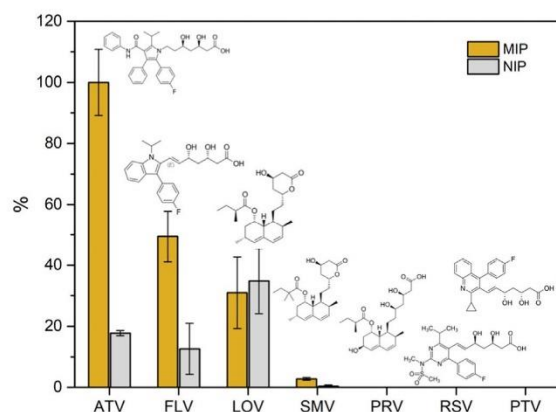


Fig. 8. The oxidation peak current percentage of $10 \mu\text{mol L}^{-1}$ ATV and its structural analogues on developed MIP and NIP sensor. The error bars indicate the standard deviation of three replicate determinations.

Table 2
Determination results of ATV concentration in spiked water samples using the developed MIP sensor^a.

Sample	[ATV] _{add} , $\mu\text{mol L}^{-1}$	[ATV] _{det} , $\mu\text{mol L}^{-1}$	Recovery, %
River water	0		
	0.100	0.102	102
	0.500	0.493	98.7
Spring water	0		
	0.100	0.092	92.4
	0.500	0.454	90.8
Tap water	0		
	0.100	0.104	104
	0.500	0.464	92.8

^a [ATV]_{add}: ATV concentration added to the sample; [ATV]_{det}: ATV concentration determined in the sample.

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4. Conclusions

A simple, low-cost and disposable selective MIP electrochemical sensor was proposed for the first time to detect ATV. Computational studies represented a valuable approach to the development of environmental responsible MIP design and showed its excellent correlation with experimental results to acquire an efficient molecular imprinting process. The successful and easy combination of MIP with a SPCE offers the possibility of portability, which is advantageous and desired in environmental analysis. Within this concept and under optimum operational conditions, the proposed MIP sensor exhibited high binding affinity towards ATV molecules with good sensitivity as revealed by an LOD of $0.049 \mu\text{mol L}^{-1}$ and an LOQ of $0.162 \mu\text{mol L}^{-1}$ achieved over a concentration range from 0.05 to $2 \mu\text{mol L}^{-1}$ without an exterior redox probe. Excellent selectivity for ATV compared to interfering statins, such as simvastatin, is one of the merits of the sensor. Its reliability was proved in spiked tap water, spring water and water collected from river with recovery values ranging from 92 to 104%. Notably, its application was done without requiring advanced equipment and complicated sample treatment. Additionally, as a result of reproducibility studies, RSD value was found to be 6.1% ($n = 4$) indicating that the obtained results had good accuracy and precision. The research outcomes showed that this MIP sensor has clear advantages and can hold a promise to ATV detection in environmental waters.

CRediT authorship contribution statement

Patrícia Rebelo: Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **João G. Pacheco:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. **Iuliia V. Voroshyllova:** Software, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **André Melo:** Software, Data curation, Writing – review & editing. **M. Natália D.S. Cordeiro:** Software, Writing – review & editing, Supervision. **Cristina Delerue-Matos:** Writing – review & editing, Supervision, All authors have read and approved the final manuscript.

Declaration of competing interest

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

Acknowledgements

Patrícia Rebelo (SFRH/BD/132384/2017) and João Pacheco (SFRH/BPD/101419/2014) are grateful to Fundação para a Ciência e a Tecnologia (FCT) and European Union (EU) for their grants, financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu (FSE) and Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). The work was supported by the project Farmasense (39957) that was funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020) and the Fundo Europeu de Desenvolvimento Regional (FEDER). This work was further supported by UID/QUI/50006/2020 (LAQV-REQUIMTE) with funding from FCT/MCTES through national funds. The authors would like to thank the EU and FCT/UEFISCDI/FORMAS for funding, in the frame of the collaborative international consortium REWATER financed under the ERA-NET Cofund WaterWorks 2015 Call. This ERA-NET is an integral part of the 2016 Joint Activities

developed by the Water Challenges for a Changing World Joint Programme Initiative (Water JPI).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2021.339410>.

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Supporting information

A simple electrochemical detection of atorvastatin based on disposable screen-printed carbon electrodes modified by molecularly imprinted polymer: experiment and simulation

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Table S1. Calculated binding energies obtained between ATV and different functional monomers at M06-2X/6-31+G* level.

Complex	$\Delta E/\text{kJ mol}^{-1}$
ATV-(ABA)	-76.064
ATV-(Indole)	-74.359
ATV-(4-Aminothiophenol)	-70.494
ATV-(Carbazole)	-65.613
ATV-(Mercaptobenzimidazole)	-63.733
ATV-(Pyrrole)	-56.049
ATV-(Aniline)	-51.336
ATV-(Phenol)	-46.428
ATV-(o-Phenylenediamine)	-39.675
ATV-(1-vinylimidazole)	-36.214

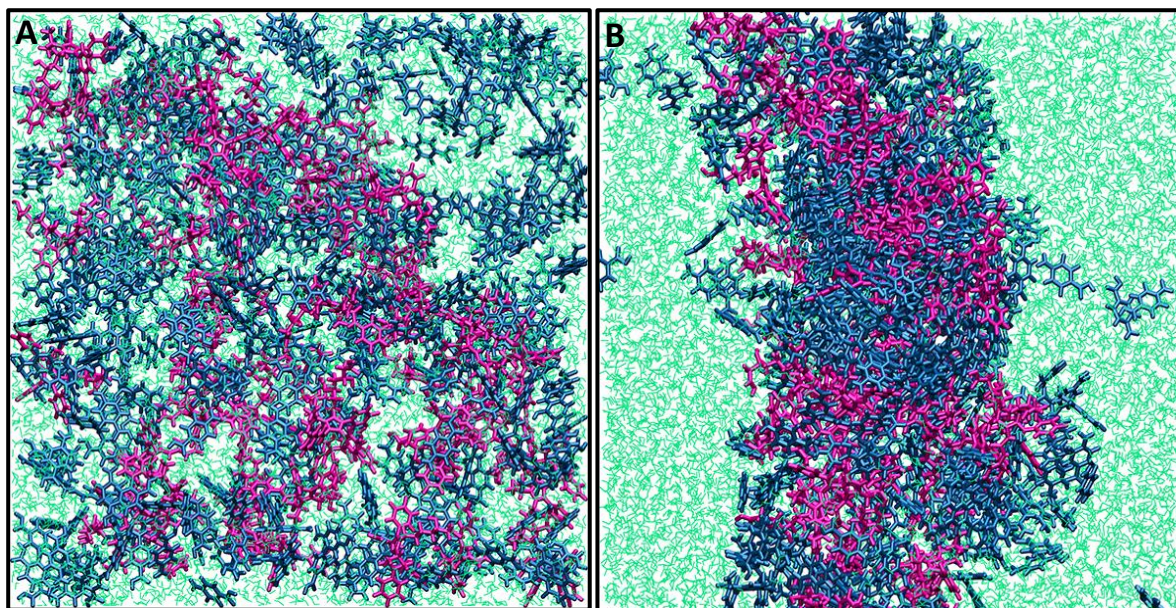
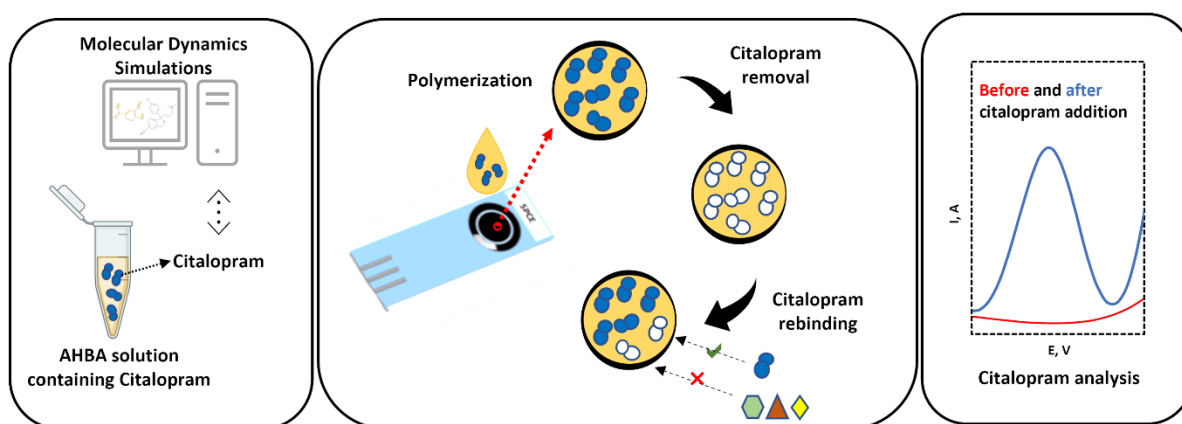


Fig. S1. Snapshots of the initial simulation box (A) and of the same box after conducting the MD simulations (B). The template ATV molecules are shown as purple, functional monomer ABA as blue and solvent as green structures.

3.5 Computational modelling and sustainable synthesis of a highly selective electrochemical MIP-based sensor for citalopram detection

Molecules 27 (2022) 3315



Statement of contribution

The contribution of the first author, Patrícia Rebelo, in this work includes the literature review, investigation, methodology and writing of the original draft.

Article

Computational Modelling and Sustainable Synthesis of a Highly Selective Electrochemical MIP-Based Sensor for Citalopram Detection

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Abstract: A novel molecularly imprinted polymer (MIP) has been developed based on a simple and sustainable strategy for the selective determination of citalopram (CTL) using screen-printed carbon electrodes (SPCEs). The MIP layer was prepared by electrochemical in situ polymerization of the 3-amino-4 hydroxybenzoic acid (AHBA) functional monomer and CTL as a template molecule. To simulate the polymerization mixture and predict the most suitable ratio between the template and functional monomer, computational studies, namely molecular dynamics (MD) simulations, were carried out. During the experimental preparation process, essential parameters controlling the performance of the MIP sensor, including CTL:AHBA concentration, number of polymerization cycles, and square wave voltammetry (SWV) frequency were investigated and optimized. The electrochemical characteristics of the prepared MIP sensor were evaluated by both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques. Based on the optimal conditions, a linear electrochemical response of the sensor was obtained by SWV measurements from 0.1 to 1.25 $\mu\text{mol L}^{-1}$ with a limit of detection (LOD) of 0.162 $\mu\text{mol L}^{-1}$ (S/N = 3). Moreover, the MIP sensor revealed excellent CTL selectivity against very close analogues, as well as high imprinting factor of 22. Its applicability in spiked river water samples demonstrated its potential for adequate monitoring of CTL. This sensor offers a facile strategy to achieve portability while expressing a willingness to care for the environment.

Keywords: antidepressants; environmental pollution; molecularly imprinted polymer; screen-printed electrode; computational studies



Citation: Rebelo, P.; Pacheco, J.G.; Voroshylova, I.V.; Seguro, I.; Cordeiro, M.N.D.S.; Delerue-Matos, C. Computational Modelling and Sustainable Synthesis of a Highly Selective Electrochemical MIP-Based Sensor for Citalopram Detection. *Molecules* **2022**, *27*, 3315. <https://doi.org/10.3390/molecules27103315>

Academic Editor: Amit Das

Received: 7 April 2022

Accepted: 19 May 2022

Published: 21 May 2022

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1. Introduction

Antidepressant drug consumption has been rapidly growing every year. According to the Organization for Economic Cooperation and Development (OECD) statistics, the use of antidepressants doubled in 28 OECD countries between 2000 and 2017 [1,2]. Currently, their consumption levels are breaking records, and increased inputs of these compounds into environmental compartments have been reported by the scientific community [2–7]. In the future, some authors believe that this problem of emerging concern will be amplified as an immediate effect of the COVID-19 pandemic on the mental health of the communities [5,8,9].

Citalopram (CTL) is one of the most important antidepressants of the serotonin reuptake inhibitors (SSRIs) class, which is widely used throughout the world [5,10]. Its mechanism of action is based on blocking the reuptake of the neurotransmitter serotonin into nerve terminals of donor cells, thereby potentiating the serotonergic activity [11,12].

This compound is a racemic mixture of R(−) and S(+) enantiomers, but its desired pharmacological effect appears to reside mainly in the S-enantiomer [13,14]. In comparison with the previously introduced tricyclic antidepressants, CTL offers advantages owing to its therapeutic profile, superior acceptability, and safety, which justifies its popularity in the treatment of major depressive disorders [3,15,16]. In parallel, there is limited information on its environmental fate and toxicity, raising concerns regarding the potential effects on exposed wildlife [17–19]. Therefore, the monitoring of its presence in environmental compartments is imperative.

Chromatographic techniques are the most commonly used methods to detect CTL in biological and water samples [4,20–22]. On the other hand, their evident drawbacks, such as the need for complex and expensive instruments, the long analysis time, and the use of large volumes of toxic solvents have encouraged the scientific community to design new approaches for the detection of pharmaceuticals. While electrochemical methods have become an interesting alternative due to their simplicity, fast response, and low cost, only few studies based on these techniques have been reported to date for the determination of CTL [13,23–30].

To improve the selectivity and performance of electrochemical techniques, the use of molecularly imprinted polymers (MIPs) is one of the most interesting strategies. Their integration with electrochemical sensors has attracted extensive attention on environmental monitoring [31–33]. Moreover, MIP-based electrochemical sensors are one of the great candidates as detection and quantification tools, due to their chemical/mechanical stability, high selectivity and sensitivity, ease of preparation, and the possibility of in situ and real-time measurements [33].

Among the different approaches applied in MIP synthesis, electropolymerization provides a very simple and rapid method (one-step synthesis) to immobilize polymeric films on the precise location of the electrode surface with good adherence [34]. Notably, electropolymerization allows the achievement of a more sustainable MIP concept, as this method does not require a crosslinker and an initiator, which is conventionally used in the traditional preparation of MIPs [35]. At the same time, small sample/reagent volumes can be used, especially when the MIP is electropolymerized on the surface of the miniaturized screen-printed electrodes (SPEs) [36,37]. Currently, sustainable chemistry, also known as green chemistry, has been the focus of many research fields aiming to minimize the negative impact of chemical products and processes on the environment [38]. Reducing the quantity of organic solvents used as well as the waste generated is a key action for sustainable chemistry [39]. Following this direction, the literature shows few examples of environmentally-friendly practices, involving green technologies and alternative solvents, that are employed for MIP sensors development [40]. Therefore, computational studies, which are the most relevant tools to reduce the synthesis of novel MIPs by trial and error, have been used by researchers for preliminary exploration of molecular imprinting processes [41,42]. Appropriate simulations and computations have led to new methods for describing, predicting, and analyzing the complexity of molecular imprinting process without repeated laboratory experiments, an endeavor that saves time, energy, and the disposal of chemicals [42–45]. Researchers have demonstrated that computational studies based on molecular dynamics (MD) simulations can provide a realistic description of functional monomer-template interactions and, at the same time, explore the effects of different monomer ratios in the polymerization mixture [42,46].

In this research work, an attempt was made to develop a sustainable electrochemical MIP-based sensor for detection of CTL based on a simple, fast, and low-cost design. To the best of our knowledge, this is the first study that presents a voltammetric MIP sensor for the detection of this compound through screen-printed carbon electrode (SPCE) modification, which is suitable for point-of-care analysis. Phosphate buffer solution was used as a “green” solvent for electropolymerization of the CTL with the building and functional monomer 3-amino-4-hydroxybenzoic acid (AHBA), as well as solvent extraction and supporting electrolyte. By taking full advantage of the use of MD simulations, five MIP

pre-polymerization mixtures were designed and simulated to predict the most efficient MIP formulation. Validation of these simulations was experimentally complemented for subsequent analyses of their electrochemical performance. The applicability of the sensor was tested in Portuguese river water samples, highlighting its ability to be used for in situ analysis through integration with portable devices. This investigation draws attention to the importance of rational approach on sensor development.

2. Materials and Methods

2.1. Reagents and Solutions

The 3-amino-4-hydroxybenzoic acid (AHBA, 97%), citalopram hydrochloride (CTL), potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihydrate, fluoxetine (FLX), and carbamazepine (CBZ) were acquired from Sigma-Aldrich. Citalopram propionic acid (CTL-PA), citalopram N-oxide (CTL-NO), didemethylcitalopram (DD-CTL), and demethylcitalopram (D-CTL) were supplied by H. Lundbeck (Copenhagen, Denmark). Stock solutions of CTL (5 mmol L^{-1}) were prepared in phosphate buffer solution (0.1 M, pH 7) and used to prepare less concentrated standards. Phosphate buffer solution was prepared with a mixture of KH_2PO_4 and K_2HPO_4 (Riedel-de-Haën). Ultra-pure water (with resistivity value of $18.2 \text{ M}\Omega\text{-cm}$) obtained from a Millipore (Simplicity 185) water purification system was used throughout the work. The water samples were collected from one Portuguese river. All of the chemicals were used as supplied.

2.2. Apparatus and Equipment

In this work, all of the electrochemical techniques were performed using SPCEs (DRP-110, Methrom, Spain). The configuration consists of a three-electrode arrangement printed on an alumina substrate with a circular carbon working electrode (WE, 4 mm ϕ), a carbon auxiliary electrode (CE), and a silver pseudo-reference electrode (RE). Cyclic voltammetry (CV) and square wave voltammetry (SWV) were carried out on a Metrohm Autolab PGSTAT 204 potentiostat/galvanostat controlled by NOVA 1.11 software. Electrochemical impedance spectroscopy (EIS) was conducted using a Metrohm Autolab PGSTAT 128 N potentiostat/galvanostat. This device was controlled by NOVA 1.6 software. To measure pH, a Crison pH meter (model micropH 92002) with a combined glass electrode was used. All of the experiments were conducted at room temperature.

2.3. Fabrication of the MIP-CTL Sensor and Electrochemical Analysis

To produce a homogeneous and thin MIP-CTL film, 40 μL of polymerization solution, containing 0.5 mmol L^{-1} CTL and 1.5 mmol L^{-1} AHBA in phosphate buffer, was gently dropped on the surface of the SPCE. Electropolymerization was performed by 10 consecutive CV cycles from -0.2 to 1.5 V at the scan rate of 100 mV s^{-1} . Then, the electrodes were carefully washed with deionized water and air dried. To extract CTL molecules from the polymeric matrix, the modified SPCEs were subjected to 5 CV scans between -0.5 and 1.2 V at 100 mV s^{-1} in phosphate buffer. The non-imprinted polymer (NIP sensor) was prepared using the same procedure, but in the absence of CTL as a template in the electropolymerization process.

For rebinding of CTL molecules, the MIP sensor was incubated with 40 μL CTL solution for 15 min. Then, the electrodes were rinsed, and their electrochemical response was evaluated by SWV measurements in phosphate buffer over a potential interval from 0.4 to 1.2 V with a frequency of 20 Hz , a potential step of 5 mV s^{-1} , and a pulse amplitude of 50 mV s^{-1} . All of the measurements were performed in phosphate buffer as redox probe to study the electrochemical response of the prepared MIP sensors and were made in triplicate.

Under optimized conditions, CV and EIS experiments were employed to characterize the different steps of MIP-CTL construction. For this purpose, the current and resistance variations of 2.5 mmol L^{-1} $[\text{Fe}(\text{CN})_6]^{3-/4-}$ prepared in 0.1 M KCl were evaluated. The CVs were scanned at 100 mV s^{-1} between -0.2 and 0.5 V . EIS assays were operated at a

potential of 0.2 V over the frequency range of 0.1 to 10 Hz with 50 frequencies distributed logarithmically and a signal amplitude of 10 mV. EIS experimental data were acquired by Nyquist plots fitted based on Randles equivalent electrical circuit model.

2.4. Computational Setup

The MD study was performed in a similar manner to our previous works [43–45]. In short, all of the MD simulations were carried out with the GROMACS 5.1.4 software package [47]. For CTL and AHBA molecules, the general amber force field (GAFF) was used as received from the ANTECHAMBER module (including partial charges) [48]. During the experiments, the synthesis of this MIP occurs in phosphate buffer (see Section 2.3). For the computational study, all of the molecular species were placed in water to represent the solvent. Solvent water, based on literature reports [45,49,50], was selected for modelling by the TIP3P FF [51]. Several concentrations of functional monomer in pre-polymerization mixtures were investigated (see Section 3.1; Table 1) to identify the most favorable CTL:AHBA ratio. Therefore, the number of interaction sites in the systems varied from 33,150 for 1:1 template:monomer ratio to 37,650 for 1:6 ratio. Although the pharmacological effect of citalopram is associated with the S(+) enantiomer, in the most common commercial form both enantiomers are present in a racemic mixture. Therefore, we decided to emulate this composition in our simulations.

Table 1. Composition of the studied pre-polymerization mixtures.

Mixture	Molar Ratio (CTL:AHBA)	Number of Molecules				Total Number of Interaction Sites
		S(+)-CTL	R(−)-CTL	AHBA	H ₂ O	
1	1:1	25	25	50	10,000	33,150
2	1:2	25	25	100	10,000	34,050
3	1:3	25	25	150	10,000	34,950
4	1:4	25	25	200	10,000	35,850
5	1:6	25	25	300	10,000	37,650

For the systems initiation, all of the necessary molecules were placed randomly into cubic simulation boxes using the PACKMOL code [52]. After energy minimization with the steepest-descent algorithm, two short pre-equilibrations in the canonical (for 100 ps at 100 K) and isothermal-isobaric (for 500 ps at 298 K) ensembles were performed. This was followed by an annealing procedure during 1 ns in the *NVT* ensemble, consisting of slowly heating the system up to 370 K and then slowly cooling it down to 298 K, which allowed us to overcome possible energy barriers. During all of the above-described MD steps, integration times of 0.5 fs were employed and no bond constraints were applied. Then, the equilibration (for 1 ns in *NpT*) and production (for 20 ns in *NVT*) followed at 298.15 K and 1 bar (only equilibration) conditions. At these two last stages, a time step of 1 fs was used and the bonds with hydrogens were constrained with the LINCS algorithm [53]. To maintain constant temperature, the Nosé–Hoover thermostat [54,55] was applied throughout the work with a coupling constant of 2.0 ps. Moreover, to maintain the pressure at a fixed level, where applicable, the Parrinello–Rahman barostat [56] was employed with a coupling constant of 8.0 ps. The leap-frog algorithm [57] was used for integrating the equations of motion. Periodic boundary conditions were applied in all directions with a cut-off of 1.2 nm set for both energy and pressure.

GROMACS in-built tools were employed to calculate the density, *d*, self-diffusion coefficients, *D*, radial distribution functions (RDFs), and hydrogen-bonding (H-bond) interactions. Self-diffusion coefficients were only evaluated after examining the attainment of a diffusive regime. To accomplish this, the parameter β was computed for each species and each simulation cell, as comprehensively described elsewhere [58–61]. The GRACE software was used for the numerical integration of RDFs for coordination numbers (CNs) assessment. When applicable, the error (i.e., deviation of a given simulated value from an experimental

one) of a physical property was estimated as follows: $(|X_{\text{exp}} - X_{\text{sim}}|) / X_{\text{exp}} \times 100\%$, where X is the property obtained experimentally, X_{exp} , or computationally, X_{sim} . A schematic diagram of the simulated species, with the atom numbering used throughout this work, is provided in Figure 1.

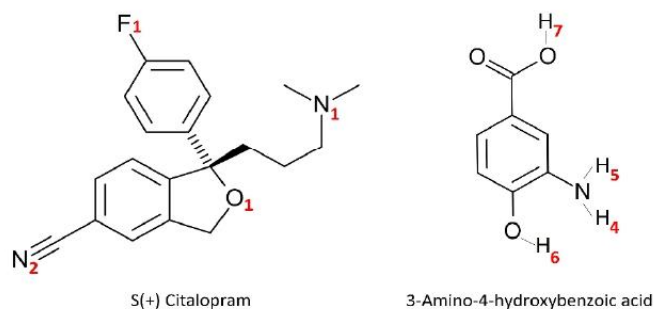


Figure 1. Schematic representation of S(+) citalopram, CTL with the chiral center indicated, and 3-amino-4-hydroxybenzoic acid (AHBA), along with some atom types, used throughout this work.

3. Results and Discussion

3.1. MD Simulations

During the preparation of a MIP sensor, one of the most important factors to consider is the interaction between the template and the functional monomer, as this significantly impacts the number of selective binding sites created on the polymeric matrix. In this context, finding the optimal molar ratio of template to the functional monomer is of great importance. Therefore, to evaluate the effect of the polymerization mixture components concentration as well as to gain a better insight into the possible interactions that occur between CTL and AHBA during MIP synthesis, MD simulations were carried out. Five different pre-polymerization mixtures were tested with varying concentrations of AHBA (Table 1).

To achieve high accuracy in reproducing experimental polymerization conditions, all of the mixtures were simulated using water as solvent. At the same time, CTL was considered as a racemic mixture during the design of the initial simulation box. In addition, as can be seen in Table 1, proportions of equal molecules of S(+) and R(−) enantiomers were included in the mixtures composition. Following preliminary tests, it was found that S(+) CTL and R(−) CTL do not present notable differences in local structure. Indeed, radial distribution functions, $g(r)$, revealed virtually identical interactions of each CTL enantiomer with AHBA (see Figure S1), suggesting that the developed MIP sensor will not be able to distinguish between CTL enantiomers. Further analyses will help in the treatment of both CTL enantiomers.

3.1.1. Density and Self-Diffusion Coefficients

To control the quality of the simulations and the employed FF models, the density and self-diffusion coefficients were evaluated and listed in Table 2.

Table 2. Density (d) and self-diffusion coefficients (D) of the studied pre-polymerization mixtures.

Mixture	$d, \text{kg m}^{-3}$	$D \times 10^9, \text{m}^2 \text{s}^{-1}$		
		CTL	AHBA	H ₂ O
1	1009.43	0.13 ± 0.02	0.28 ± 0.01	5.21 ± 0.11
2	1021.88	0.11 ± 0.00	0.20 ± 0.02	4.87 ± 0.10
3	1032.86	0.12 ± 0.01	0.24 ± 0.02	4.53 ± 0.06
4	1043.96	0.03 ± 0.04	0.13 ± 0.00	4.32 ± 0.04
5	1064.54	0.06 ± 0.01	0.16 ± 0.03	3.94 ± 0.04

As expected, the results showed a good correlation between the increase in the systems density with the increase in the AHBA molar fraction. Regarding the values of simulated self-diffusion coefficients, a similar diffusive behavior between CTL and AHBA can be noticed. The diffusion values of these molecules decline gradually from mixture 1 to mixture 5, with the exception of a slight increase registered in mixture 3. Taking into account that CTL needs to present enough mobility to allow its diffusion into and out of the MIP binding sites [62], mixtures 4 and 5 seem to show an unfavorable performance. In contrast, valuable results are obtained for both mixtures 1 and 3, as these have higher values of CTL diffusion coefficients. However, it is necessary to collect more data and evaluate other factors to infer the suitability of these mixtures to the experiment.

3.1.2. Local Structure Analysis: RDFs and H-Bonds

RDFs were calculated to analyze the predictions for binding of CTL and AHBA on a molecular level in different pre-polymerization mixtures. Interactions between specific parts of these compounds were studied and discussed here only for the most active atoms (depicted in Figure 1), which are identified in a series of initial tests. The resulted RDFs are shown in Figure 2. All of the studied mixtures demonstrated favorable CTL-AHBA interactions, where the well-defined peaks can be observed at about 0.2 nm, suggesting the establishment of a hydrogen-bonding network.

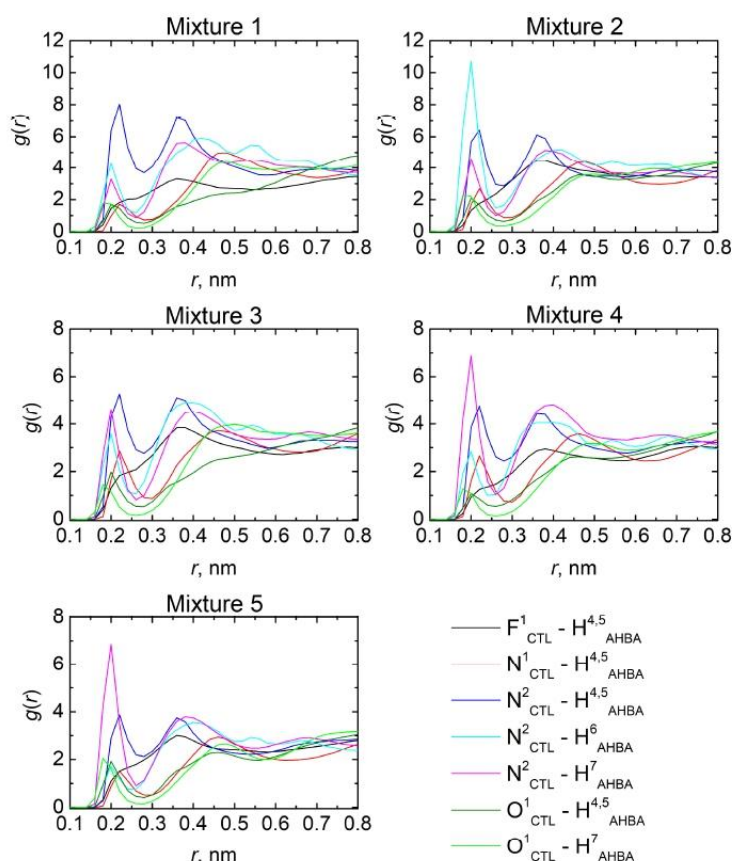


Figure 2. Radial distribution functions, $g(r)$, between specific sites of CTL and AHBA molecules in the studied pre-polymerization mixtures from the results of molecular dynamics simulations.

Notably, AHBA displays a good affinity for CTL by the tertiary amine (N^1 , see Figure 1), the single oxygen-linked group (O^1), as well as for the cyan group (N^2) and fluor atom (F^1) of the two different substituted benzene rings. In parallel, the extent of the interaction of AHBA is carried out by four hydrogens atoms, namely the two hydrogens of the amine group (H^4 and H^5), and the two hydrogens of hydroxyl groups (H^6 and H^7). Undoubtedly, the interactions observed between N^2_{CTL} and $H^{4,5,6,7}_{AHBA}$ have an outstanding role in comparison with the other interactions. This finding is supported by the height of these characteristic peaks, which are more or less strengthened in different mixtures. However, following the results mentioned above, mixture 3 was judged to present the most balanced complex, which is supported by its defined peaks with a more homogeneous interaction distribution between CTL and AHBA molecules. Therefore, this can be explained by the fact that AHBA molecules are more uniformly distributed around CTL when compared with the other mixtures, which can be particularly advantageous to obtain a strong imprinting effect.

Based on these results, the extent of hydrogen-bonds (H-bonds) formation in each mixture was analyzed following the framework of Luzar and Chandler geometrical criteria [63], according to which a hydrogen bond can be considered if the distance between the oxygens of two different molecules is less than 3.5 Å and, simultaneously, the angle formed by O-H...O bond is less than 30°. Table 3 summarizes the total amount of H-bonds per mixture and the number of interactions per CTL molecules. A notable increase in H-bonds was registered with the increase in AHBA concentration, which tends to stabilize after mixture 3. This result is rationalized as a type of saturation of CTL-AHBA binding capacity. Due to the fact that hydrogen bonds have a key role in the imprinting process with direct influence on MIP efficacy and taking into account the performance of all the addressed pre-polymerization mixtures in terms of density, self-diffusion, and RDFs, it can be assumed that mixture 3 exhibits the most appropriate behavior. Consequently, the most promising ratio between CTL and AHBA with potential to create favorable imprinting sites during the electropolymerization process is 1:3.

Table 3. Total average number of hydrogen bonds at different pre-polymerization compositions and their corresponding interactions as a function of CTL molecules.

Mixture	Total H-Bonds	Interactions Per 50 CTL Molecules
1	8.96	9
2	43.58	22
3	67.45	22
4	70.22	18
5	78.54	16

3.2. Electrochemical Preparation of MIP-CTL Sensor and Its Recognition Abilities

In this work, the molecular imprinting of CTL molecules on SPCE surface was performed by simple electropolymerization of an optimized phosphate buffer solution containing 0.5 mmol L⁻¹ CTL as template and 1.5 mmol L⁻¹ AHBA as functional monomer. In addition to its efficient interaction with CTL, AHBA was employed due to its ability to provide a sustainable design of MIP, since this aromatic functional monomer has been considered as one candidate for bio-based monomers [64]. Moreover, it is highly soluble in common solvents and can be easily obtained by a microorganism (*Streptomyces griseus*), which promotes a more responsible mass-production process [64,65].

Figure 3A shows the resulting CV curves of electropolymerization process, which is performed by 10 consecutive cycles between −0.2 and 1.5 V. Here, a poly(AHBA) film with trapped CTL molecules was created. As seen from the figure, two oxidation peaks at about 0.3 and 0.98 V were registered. While the first peak can be attributed to the electrochemical oxidation of the AHBA at the SPCE surface, the second peak predicts the oxidation of the embedded CTL molecules during MIP film formation. Consequently, significant differences were observed between the MIP and NIP film growth (Figure 3B).

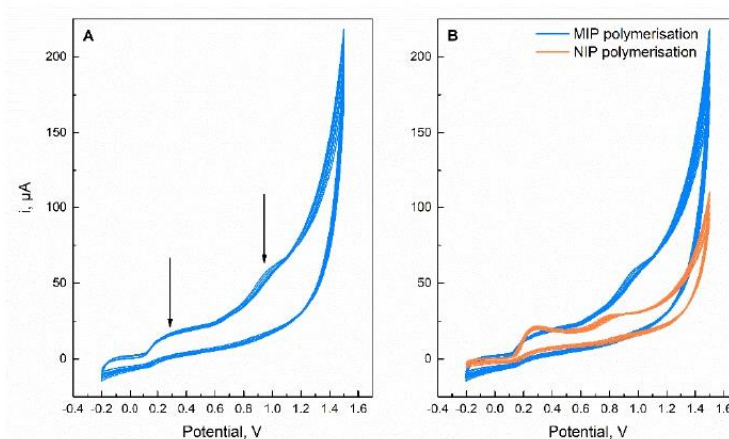


Figure 3. (A) Cyclic voltammograms of the MIP SPCE modification with 1.5 mmol L⁻¹ ABA and 0.5 mmol L⁻¹ ATV in buffer solution and (B) comparison of cyclic voltammograms building in MIP and NIP sensors.

In addition to the scans of the present NIP film electro-generation, the electrochemical oxidation peaks of AHBA are more pronounced than MIP, i.e., the oxidation peaks at 0.3 and 0.8 V. However, no oxidation peak was detected at 0.98 V (Figure 3B). These findings should stem from the absence of CTL molecules that in the case of MIP synthesis triggered the amplification of the current response and did not allow for a complete visualization of the AHBA oxidation peak at 0.8 V, but rather, highlighted the oxidation peak of CTL molecules at 0.98 V.

Following the preparation of the polymeric film, the imprinting effects on sensor performance, including the incorporation of CTL molecules in the MIP film, as well as its elution and subsequent rebinding interaction, were evaluated by SWV measurements in phosphate buffer solution. The response profile on NIP and MIP-CTL SPCE recorded at a potential window from 0.4 to 1.2 V is shown in Figure 4. Therefore, a remarkable difference is noticeable in the electrochemical behavior of the resulting NIP and MIP film growth. As depicted in Figure 4A, a broad oxidation peak at about 0.8 V was exclusively observed in the MIP sensor, demonstrating that CTL molecules were successfully entrapped in the polymeric MIP layer deposited on the SPCE surface.

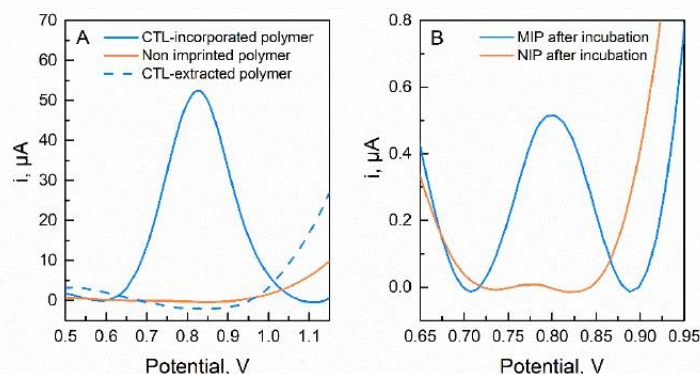


Figure 4. SWV voltammograms in 0.1 M of phosphate buffer (pH = 7) obtained after (A) MIP (0.5 mmol L⁻¹ CTL, 1.5 mmol L⁻¹ AHBA) and NIP (1.5 mmol L⁻¹ AHBA) polymerization and extraction of CTL molecules; (B) MIP and NIP sensing responses after 15 min of 1 μmol L⁻¹ CTL incubation.

Commonly, to remove the trapped molecules from MIP and leave the rebinding cavities, solvent extraction is adopted. However, from a sustainable development perspective, this procedure was replaced by a fast and simple electrochemical overoxidation process. In this work, the extraction of CTL molecules from poly(AHBA) matrix was achieved by scanning the potential between -0.5 and 1.4 V in phosphate buffer solution, without the use of organic solvents. The resulting CV voltammograms (Figure S2) revealed that the increase in the cycle number displayed a decreasing peak current intensity until no obvious peak appeared after five cycles. This behavior suggests that CTL template molecules were removed from the imprinted polymer, as it was confirmed by SWV analysis, where no current peak was again observed (Figure 4B). Next, the rebinding of $1 \mu\text{mol L}^{-1}$ CTL showed a significant difference between the intensity of the obtained oxidation peaks from MIP and the corresponding NIP. The higher binding capacity of the MIP sensor is clearly attributed to the imprinting effect, which allowed it to adsorb a large amount of target molecules. Consequently, all of these outcomes prove the successful construction of MIP layer with complementary cavities to CTL molecules.

3.3. Optimization of Experimental Parameters in Preparation and Detection Process

The molar ratio between the template and functional monomer, the number of cycles during the electropolymerization process, and the frequency of SWV measurements are critical parameters that are responsible for the characteristics regulation and performance of the designed MIP sensor. To achieve the maximum current response and ensure an optimal selectivity of the sensor, the influence of these parameters on electrochemical response was evaluated in both MIP and NIP sensors. The results are illustrated in Figure 5.

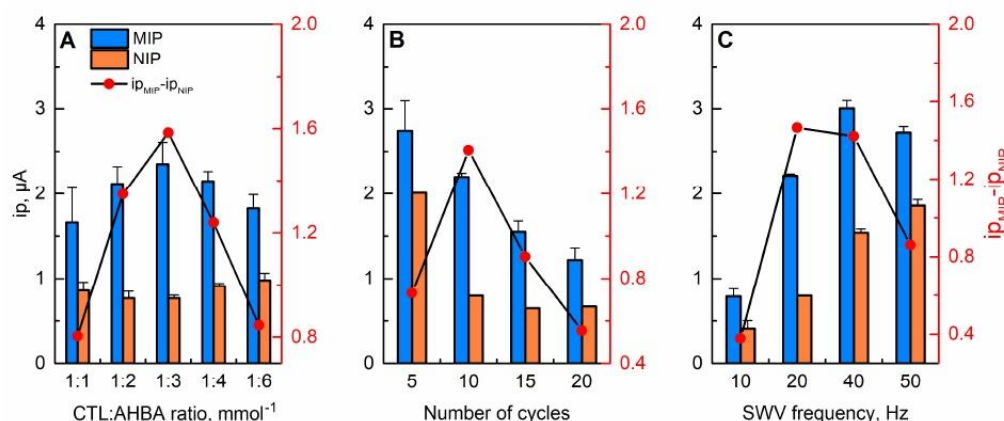


Figure 5. Effects of (A) molar ratio of CTL to AHBA, (B) number of polymerization scan cycles, and (C) frequency applied during SWV on sensing responses of MIP and NIP sensors toward $10 \mu\text{mol L}^{-1}$ CTL.

Taking into consideration the results predicted by the described above MD simulations, the same conditions of template-functional monomer molar ratio were also experimentally studied to validate the accuracy of the proposed method. For this, the concentration of 0.5 mmol L^{-1} CTL remained constant and five different ratios of AHBA were tested (0.5 , 1 , 1.5 , 2 , and 3 mmol L^{-1}), with varying ratios of CTL-AHBA. Notably, the NIP sensor was developed in the absence of CTL molecules and replaced by the phosphate buffer solution. As it can be seen in Figure 5A, the response signal of MIP sensor to CTL increases up to 1:3 ratio, suggesting that the higher amount of functional monomer provides more effective binding sites for CTL rebinding. However, when the molar ratio exceeded 1:3, a downward trend was observed in MIP sensor, which is possibly due to the presence of an excess amount of monomer that hinders the proper extraction and rebinding of CTL molecules.

At the same time, no significant differences in the response signal of the NIP sensor were registered. However, at very high AHBA concentrations, there is a clear decrease in the difference between the peak current intensity of the MIP and NIP sensors and, consequently, a decrease in sensor sensitivity. These findings are in line with the predictions proposed by the computational studies and confirm the optimum ratio for the template and functional monomer of 1:3.

To investigate the effect of the polymeric film thickness and, consequently, the diffusion rate of CTL, the effect of the number of cycles applied during the electropolymerization procedure was explored under variations of 5, 10, 15, and 20 scan cycles (Figure 5B). As it can be seen in Figure 5B, this parameter has a strong influence on the sensor performance. Curiously, there is a gradual decrease in the peak current intensity of the MIP sensor with the number of cycles, indicating that the diffusion of CTL molecules is impaired by thicker sensing films. On the other hand, for comparison with the response of the NIP sensor, it was found that five polymerization cycles were unable to produce enough and selective imprinted cavities, as the difference between the electrochemical response of NIP and MIP sensors toward CTL is very low. The highest difference was obtained by applying 10 scan cycles in the polymerization step. For this reason, 10 cycles were selected for further experiments.

To obtain the best analytical performance, the effect of the frequency parameter on SWV measurements was also studied by increasing the frequency from 10 to 50 Hz (Figure 5C). The results presented in Figure 5C show that with the increase in SWV frequencies, a significant enhancement in the electrochemical signal of MIP sensor until a maximum of 40 Hz occurs. In MIPs, the decrease in the current response at frequencies higher than 40 Hz can be the reflection of insufficient time for diffusion of CTL molecules within imprinted cavities, thus limiting the electrochemical activity. Considering the maximum difference between the responses of the NIP and MIP sensors, a frequency of 20 Hz was used in this work as an optimum value of SWV frequency. Additionally, the developed MIP sensor was subjected to time incubation studies. It was found that 15 min of incubation period was optimal for obtaining adequate rebinding of CTL on the MIP sensor.

3.4. Electrochemical Characterizations of the Stepwise MIP-CTL Construction

The successful construction of MIP-CTL sensor was monitored step by step, analyzing the changes in the electron transport properties of the sensing surface. For this purpose, CV and EIS measurements were performed in a 0.1 M KCl solution containing $2.5 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3-/4-}$ as redox probe. The electrochemical behavior of the bare SPCE and modified electrodes is shown in Figure 6.

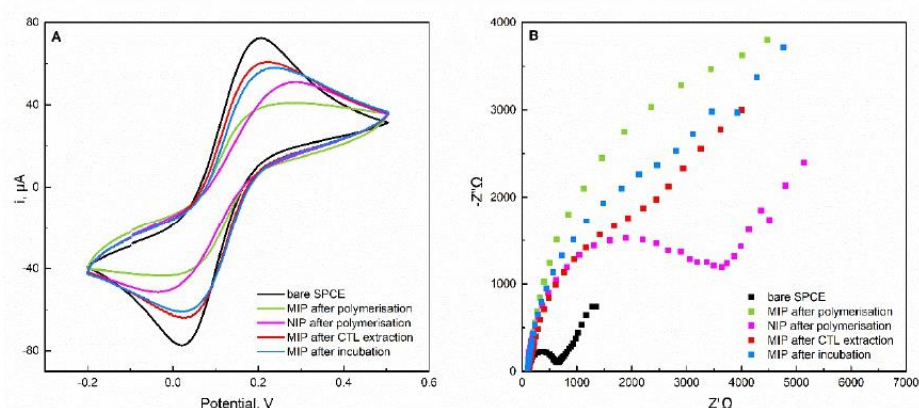


Figure 6. (A) Cyclic voltammograms and (B) impedance plots of a $2.5 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M KCl solution corresponding to the stepwise construction of the sensor.

As expected, the bare SPCE exhibits the highest redox characteristic peaks for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in voltammogram (Figure 6A). When the poly(AHBA) film modified the SPCE surface, both NIP and MIP sensors registered a decrease in the redox peak current of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a result of the non-conductive polymer produced at SPCE surface that blocked the electron transfer. Distinctly, the MIP sensor shows a remarkable decrease in the peak current intensity, which is consistent with the entrapment of CTL in the polymeric film. The conductivity of the MIP sensor was recovered by extracting CTL molecules, which was accompanied by a significant increase in the peak current of the redox probe. Following incubation, the redox activity at MIP SPCE once again decreases, indicating that CTL occupied some of the previously formed cavities and, consequently impeded the ion diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ through the MIP layer.

The obtained information on the electrochemical characteristics of the electrodes after different modifications was complemented by EIS analyses. In accordance with CV data, the Nyquist plots displayed in Figure 6B showed significant differences between the impedance behavior of the electrodes. Following polymerization, a large increase in the diameter of the semicircle portion compared with the bare SPCE can be observed, implying an increase in the charge-transfer resistance (R_{ct}). Therefore, the effective modification of the surface of SPCE was confirmed. Moreover, the R_{ct} presented by the MIP sensor was higher than the ones obtained with the NIP, which is ascribed to the presence of CTL acting as a barrier for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ diffusion. Furthermore, the removal of CTL molecules from MIP while leaving the channels free for the electron transfer process lowers the R_{ct} . In contrast, when the MIP sensor was incubated, an enhancement in the diameter of the semicircle was again observed due to the obstruction effect. These observations prove the successful construction of the MIP sensor.

3.5. Analytical Performance

Under the optimized experimental conditions, the MIP sensor underwent direct SWV measurements for various concentrations of CTL (from 0.1 to $1.25 \mu\text{mol L}^{-1}$) in phosphate buffer (Figure 7). The increase in the oxidation current with the corresponding increase in the CTL concentration exhibited good linearity ($R^2 = 0.992$, $n = 3$). The limit of detection (LOD, $S/N = 3$) and quantification (LOQ, $S/N = 10$) were found to be 0.162 and $0.541 \mu\text{mol L}^{-1}$, respectively.

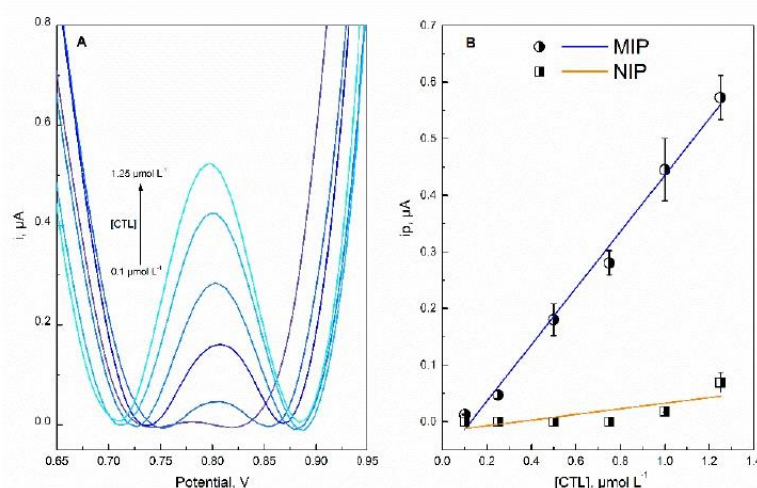


Figure 7. (A) SWV obtained from MIP response to CTL concentration in the range of 0.1 – $1.25 \mu\text{mol L}^{-1}$; (B) calibration plots for MIP and NIP sensors showing the relationship of CTL concentrations with the peak current intensity.

3.6. Selectivity and Practical Application

Selectivity is the commendable feature of an effective MIP sensor. In this work, the selectivity of the developed sensor was probed by determining the current responses of five different compounds with similarity in chemical and spatial structure to the target CTL. Additionally, the electrochemical response of a tricyclic antidepressant was evaluated. For this purpose, both NIP and MIP sensors were separately exposed to CTL, CTL-PA, CTL-NO, DD-CTL, D-CTL, FLX, and CBZ under the same conditions ($1 \mu\text{mol L}^{-1}$) for 15 min, and their corresponding SWV responses were compared. As illustrated in Figure 8, the prepared MIP sensor presents the highest sensitivity toward CTL molecules, while almost completely rejecting potential interfering analytes. The maximum imprinting factor (IF) is calculated as follows:

$$\text{IF} = ip(\text{MIP}) / ip(\text{NIP}) \quad (1)$$

where ip was as high as 22, corresponding to the peak current. No relevant analytical response was exhibited for CTL-PA, CTL-NO, DD-CTL, CTL-PA, and FLX. In addition, only a small electrochemical response was produced when the MIP sensor adsorbed these potential interferents, while no response was registered on their corresponding NIP sensors. The electrochemical response of MIP sensor to CBZ corresponds to 21% of the CTL oxidation peak, but there is a low significant difference between the intensity of MIP and NIP peak current. These findings reinforce the noticeable specific recognition of the sensor to CTL and confirm the success of the imprinting process without the need for toxic and hazardous chemicals.

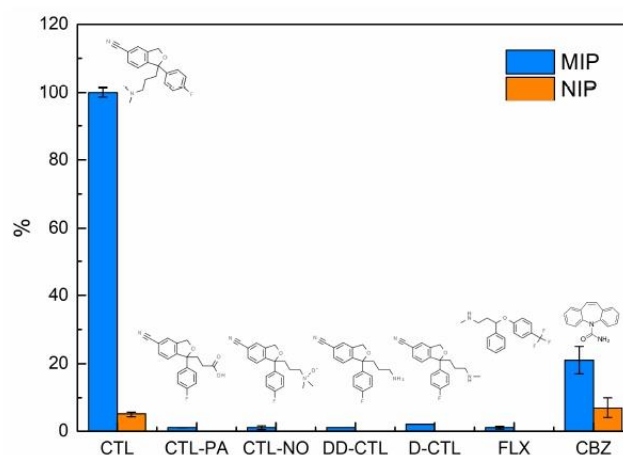


Figure 8. Electrochemical response of MIP and correspondent NIP sensor to potential interferent compounds with close structural similarities to CTL.

Subsequently, the practical applicability of the prepared sensor for recognition of CTL in water samples was evaluated using the standard addition method. The recovery study was carried out by spiking the river water samples with known concentration of CTL ($0.25 \mu\text{mol L}^{-1}$) and the analysis was performed without any pre-treatment. In these conditions, the recovery of this sensor was found to be 94.98%, indicating its sensing ability for determining CTL in water samples.

4. Conclusions

With hindsight on the design simplicity and unit activities offered by MIP implementation, the electrochemical MIP sensor developed in this work presents a valuable sustainable strategy to detect CTL. The adoption of MD simulations to optimize MIP formulation in a rational and greener method showed a good correlation with the experimental results,

proving its power as a tool to improve the molecular imprinting process, while highlighting the need to explore this area to make the use of MIPs more eco-friendly. The successful assembly of MIP on SPCE surface as well as avoiding organic solvents in the whole analytical procedure, including the CTL extraction step and reducing waste production led to a good sensitivity, as revealed by LOD of $0.162 \mu\text{mol L}^{-1}$ and LOQ of $0.541 \mu\text{mol L}^{-1}$. Furthermore, the sensor showed an interesting recovery in spiked water samples, thereby demonstrating its applicability in water. Notably, the two figures of merit for the developed MIP sensor rely on its IF of 22 and, consequently, on its excellent selectivity toward CTL molecules. Of greater significance in environmental analysis, the developed MIP sensor reconciles the greenness of the method with potential functionality as a portable and low-cost sensor to CTL detection.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/molecules27103315/s1>. Figure S1: Radial distribution functions, $g(r)$, between selected sites of R(−) CTL, S(+) CTL, and AHBA molecules; Figure S2: Cyclic voltammograms of the MIP SPCE extraction in phosphate buffer solution (0.1 M, pH 7).

Author Contributions: Methodology, investigation, formal analysis, data curation, writing—original draft, writing—review and editing, visualization, P.R.; conceptualization, methodology, formal analysis, writing—review and editing, visualization, supervision, J.G.P.; software, methodology, formal analysis, data curation, writing—original draft, writing—review and editing, visualization, I.V.V.; methodology, investigation, formal analysis, data curation, writing—original draft, writing—review and editing, visualization, I.S.; software, writing—review and editing, supervision, M.N.D.S.C.; writing—review and editing, supervision, C.D.-M. All authors have read and agreed to the published version of the manuscript.

Funding: Patrícia Rebelo (SFRH/BD/132384/2017) and João Pacheco (SFRH/BPD/101419/2014) are grateful to Fundação para a Ciência e a Tecnologia (FCT) and European Union (EU) for their grants, financed by POPH-QREN-Tipologia 4.1-Formação Avançada, funded by Fundo Social Europeu (FSE) and Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). Isabel Seguro is grateful to the project Farmasense (39957) that was funded by Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico de Portugal 2020, through the Programa Operacional do Norte (NORTE 2020) and the Fundo Europeu de Desenvolvimento Regional (FEDER). This work was further supported by UID/QUI/50006/2020 (LAQV-REQUIMTE) and the project PTDC/QUI-QAN/3899/2021 with funding from FCT/MCTES through national funds. The research was funded also by FCT and BiodivRestore Joint Call 2020–2021-European Union's Horizon 2020 research and innovation programme under grant agreement No 101003777-BiodivRestore-406/DivRestore/0002/2020-BioReset—"Biodiversity restoration and conservation of inland water ecosystems for environmental and human well-being".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not available.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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Supplementary Materials

Computational modelling and sustainable synthesis of a highly selective electrochemical MIP-based sensor for citalopram detection

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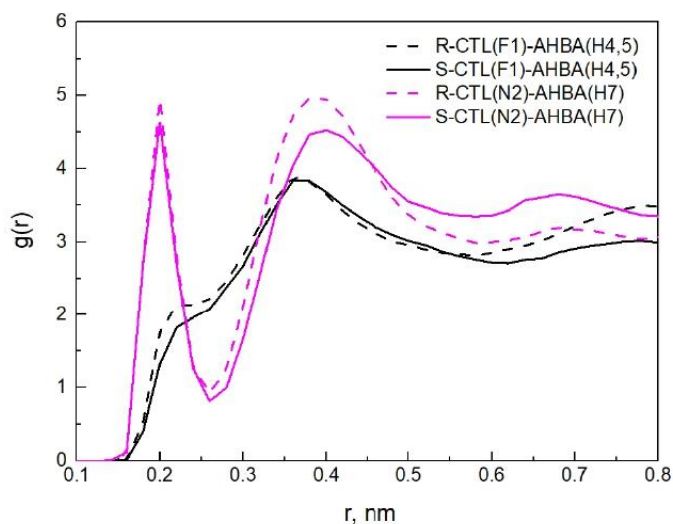


Figure S1. Radial distribution functions, $g(r)$, between selected sites of R(-)CTL, S(+)-CTL and AHBA molecules.

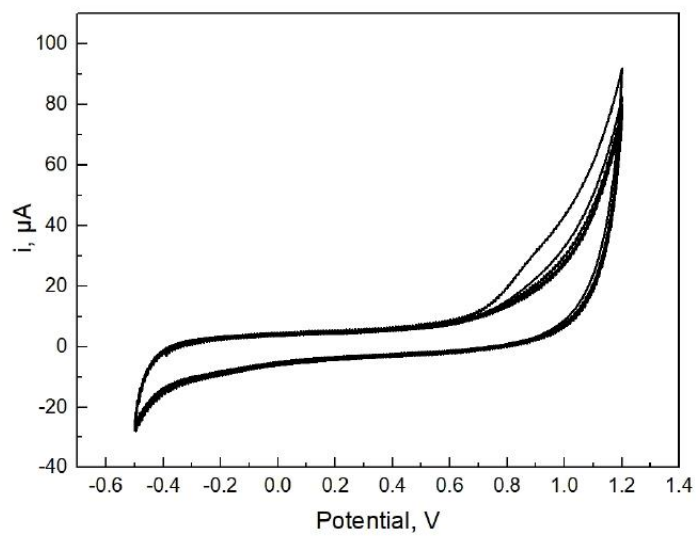


Figure S2. Cyclic voltammograms of the MIP SPCE extraction in phosphate buffer solution (0.1 M, pH 7).

Chapter

4

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

Water quality management is essential to maintain public and environmental health. This natural resource has been intensively used and explored, and its degradation has attracted much attention. At the same time, though the world sees and soon forgets, water scarcity is expected to be exacerbated as a result of climate changes, highlighting how essential it is to protect our water sources.

Given the large number of pharmaceuticals in the market, it is widely accepted that the concentration of these compounds in the aquatic environment will continue to increase. It is crucial to know their pathways, destination and effects, as well as to determine the removal efficiency through WWTPs before and after treatment. However, monitoring pharmaceuticals in environmental waters remains a challenge. Their detection can be affected by several variables, in which one of the most prominent issues is the measurement of pharmaceuticals in water samples at trace levels. Moreover, there is a need for simple, rapid, portable and sustainable analytical methods. Accordingly, the work presented in this thesis is a step forward in developing new approaches able to overcome limitations of the current analytical techniques for monitoring, since they are able to allow for automation, miniaturisation and on site determination, shortening the time between analysis.

In the last decade, the development of MIPs-based electrochemical sensors has exploded linked with increasing concerns about emerging contaminants in the environment. Although electrochemical sensors based on MIPs recognition process have proven their potential for detection and quantification of a diversity of contaminants with excellent analytical performance, few reports have been published about the analysis of pharmaceuticals in water samples, as demonstrated by the review article included in this thesis. The article review allowed to understand the trends on electrochemical MIPs sensors construction/development applied in the detection of pharmaceuticals, as well as the research gaps on environmental monitoring.

In this context, the main aim of the presented work was the development of electrochemical MIPs-based sensors for the determination of pharmaceuticals to further application in water. Sensing systems were also designed using the principles of green chemistry. Considering that MIPs construction is strongly related with extent and trial-and-error testing, the selection of functional monomers and optimisation/knowledge of the pre-polymerisation MIPs mixtures were improved by implementing molecular modelling studies. QM and MD approaches revealed to be a promising technology for preliminary exploration of MIPs protocol (design and optimise experimental parameters). At present, there is increasing research on and application of MIPs prepared by computer molecular methods, but there is still a need for further study and development of methods

to explore all aspects of imprinting process that can aid in the design of complex MIPs systems. Computational and experimental methods applied over this work, including the transducer platforms used, contemplated important aspects to address the challenge required to minimise impacts of chemicals on the environment. They allowed to reduce the volume of samples and reagents consumed and, consequently, minimise the waste generated.

AZY, FZD, AMT, ATV and CTL were the target and the template used for the construction of simple, cost-effective and potentially portable MIPs sensors. Among the electropolymerizable functional monomers studied, ABA has suggested an interesting ability to create strong molecular interactions with different kind of pharmaceuticals. Notably, its employment can be made avoiding the use of organic solvents during the imprinting process. Regarding this, *in situ* electropolymerisation is the most advantageous method, because of its simplicity for producing ultrathin polymeric film with strong adhesion to transducer surface at a both well-controlled and fast rate. Highly reproducible MIPs can be obtained.

The surface modification of the carbon electrodes using MWCNTs and SWCNTs in MIPs preparation of FZD and AMT, respectively, played an important role in the electron transfer rate, which allowed to achieve a stronger electrochemical response signal and, consequently, a better sensitivity. The combination of nanomaterials with MIPs and sensors has created new paths to improve the performance of MIPs sensors. The detection strategy involved direct analysis, avoiding the influence of masked interferents that may be attained to indirect electrochemical sensing.

Under optimum operational conditions, the developed sensors exhibited good analytical performances and, the best sensitivity was achieved by FZD MIP sensor as revealed by a low LOD of 0.03 μM . Due to their high imprinting efficiency, the electrochemical MIPs sensors also demonstrated outstanding selected response in the presence of other similar molecules. Lastly, the recovery assays in spiked water samples displayed appropriate levels of precision without requiring advanced equipment and extensive steps of samples preparation. These experimental results highlight the potential of the developed sensors to be integrated in a useful platform for the measurement of AZY, FZD, AMT, ATV, and CTL in environmental waters. At the same time, it is also important to stress that, in most cases, the presented LODs are not sufficiently low for the direct analysis of environmental waters, especially in areas where these pharmaceuticals are found at very low trace levels. The incorporation of these sensors in flow systems or preceding the analysis with a preconcentration technique can be a good alternative to lower the LOD, avoiding the cost-, time- and labour-consuming associated with chromatographic methods.

Undoubtedly, MIPs offer superior mechanical and chemical stability, easier preparation, faster responses, and lower costs in comparison with traditional instrument techniques. Nevertheless, there are still development challenges and a lot of opportunities to be explored, even in relation to the establishment of the principles of green chemistry. As a promising technology with inherent selectivity, electrochemical MIPs sensors can find a disadvantage when the determination of several compounds is required. Thus, the development of multiplex MIP-based sensors capable of recognizing different contaminants could be useful for environmental monitoring. Future works should also include efforts to transfer the knowledge from laboratories to market. Here, MIPs sensors' reusability and optimisation of their storage conditions should be explored in order to facilitate commercial applications, where the production of MIPs in homogeneous batches is very important. Transducers based on paper have also appeared as an attractive alternative to commercial electrodes and confer a sustainable feature. Paper is a versatile sensing platform that has inspired the integration of MIPs and the union of their properties will have potential prospects in environmental monitoring.

