ANALI PAZU
Vol. 14, No. 2, pp. 39–57, December 2024

ANALI PAZU

DEVELOPMENT OF BIOMIMETIC SENSORS TO DETERMINE THE FUNCTIONALITY OF IN VITRO CELL MODELS

MATJAŽ FRANGEŽ[1], NOAH EMIL GLISIK[2], MAŠA KAISER[3], JARO VEZJAK[4], JURE TERDIN[5], MATIC ŽNIDARŠIČ[6], TANJA ZIDARIČ[7] AND TINA MAVER[8]

[1] University of Maribor, Faculty of Medicine, Maribor, Slovenia, matjaz.frangez1@student.um.si
[2] University of Maribor, Faculty of Medicine, Maribor, Slovenia, noah.glisik@student.um.si
[3] University of Maribor, Faculty of Medicine, Maribor, Slovenia, masa.kaiser@student.um.si
[4] University of Maribor, Faculty of Medicine, Maribor, Slovenia, jaro.vezjak@student.um.si
[5] University of Maribor, Faculty of Medicine, Maribor, Slovenia, jure.terdin@student.um.si
[6] University of Maribor, Faculty of Medicine, Maribor, Slovenia, matic.znidarsic@student.um.si
[7] University of Maribor, Faculty of Medicine, Maribor, Slovenia, tanja.zidaric@um.si
[8] University of Maribor, Faculty of Medicine, Maribor, Slovenia, tina.maver@um.si

CORRESPONDING AUTHOR
matjaz.frangez1@student.um.si

Science:
Medicine

Advances in biomedical science have enabled the development of in vitro models that mimic human tissues. Non-invasive real-time monitoring of these models would provide valuable insights without disrupting the cellular environment. This study explores the use of molecularly imprinted polymers (MIPs) for this purpose, focusing on insulin and lactate as biomarkers of cellular metabolism. MIP sensors were created by electropolymerizing pyrrole around insulin or lactate templates on carbon electrodes. The sensors showed high sensitivity and selectivity, with detection ranges of 20.0–70.0 pM for insulin (LOD: 2.41 pM) and 0.5–3.0 mM for lactate. These findings highlight MIPs' potential for personalized diagnostics and therapy monitoring.

Keywords:
biomimetic sensors, molecular imprinting, polypyrrole matrix, insulin, MIP sensor

https://doi.org/10.18690/analipazu.14.2.39-57.2024

Univerzitetna založba Univerze v Mariboru

ANALI PAZU
Let. 14, Št. 2, pp. 39–57, december 2024

ANALI PAZU

RAZVOJ BIOMIMETIČNIH SENZORJEV ZA DOLOČANJE FUNKCIONALNOSTI IN VITRO CELIČNIH MODELOV

MATJAŽ FRANGEŽ[1], NOAH EMIL GLISIK[2], MAŠA KAISER[3], JARO VEZJAK[4], JURE TERDIN[5], MATIC ŽNIDARŠIČ[6], TANJA ZIDARIČ[7] AND TINA MAVER[8]

[1] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, matjaz.frangez1@student.um.si
[2] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, noah.glisik@student.um.si
[3] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, masa.kaiser@student.um.si
[4] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, jaro.vezjak@student.um.si
[5] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, jure.terdin@student.um.si
[6] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, matic.znidarsic@student.um.si
[7] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, tanja.zidaric@um.si
[8] Univerza Maribor, Medicinska Fakulteta, Maribor, Slovenija, tina.maver@um.si

DOPISNI AVTOR
matjaz.frangez1@student.um.si

Sprejeto
9. 10. 2024

Recenzirano
20. 12. 2024

Izdano
23. 12. 2024

Napredek v biomedicinskih znanostih je omogočil razvoj *in vitro* modelov, ki posnemajo človeška tkiva. Neinvazivno spremljanje teh modelov v realnem času bi omogočilo dragocene vpoglede brez motenja celičnega okolja. Ta študija raziskuje uporabo molekularno vtisnjenih polimerov (MIPs) za ta namen, s poudarkom na inzulinu in laktatu kot biomarkerjih celičnega metabolizma. Senzorji MIP so bili ustvarjeni z elektropolimerizacijo pirrola okoli predloge inzulina ali laktata na karbonskih elektrodah. Senzorji so pokazali visoko občutljivost in selektivnost z razponi zaznave od 20,0 do 70,0 pM za inzulin (LOZ: 2,41 pM) in od 0,5 do 3,0 mM za laktat. Ti rezultati poudarjajo potencial MIP za uporabo v personalizirani diagnostiki in spremljanju terapij.

Znanstvena veda:
Medicina

Ključne besede:
biomimetični senzorji, molekularno vtiskanje, polipirol, inzulin, MIP senzor

University of Maribor Press

https://doi.org/10.18690/analipazu.14.2.39-57.2024

1 Introduction

Advances in biomedical science over the last decade have led to the development of sophisticated *in vitro* models, such as organ-on-a-chip (OOC) microphysiological systems, which accurately simulate the functions of human tissues and organs (Kumar et al., 2024). Although these systems are crucial for long-term studies, real-time monitoring of cell functionality remains a challenge. Existing methods, such as manual sampling or techniques with limited continuous process monitoring capabilities, are often inefficient. In this context, molecularly imprinted polymers (MIPs) present an innovative solution (Zidaric et al., 2023).

Their ability to selectively recognize specific molecules enables precise analyte detection, enhancing our understanding of cellular responses and potentially advancing the diagnosis and treatment of various diseases (Li et al., 2024).
The molecular imprinting process involves three steps: 1) the target molecule (template) forms a complex with the functional monomer, 2) the monomer polymerizes around the template in the presence of an initiator and crosslinker, creating a three-dimensional (3D) matrix, and 3) the template is removed, leaving behind specific receptor sites tailored for the re-binding of the target analyte.

MIPs can be fabricated using either covalent or non-covalent approaches, with the latter being more commonly employed due to its simplicity and versatility (Sajini & Mathew, 2021).

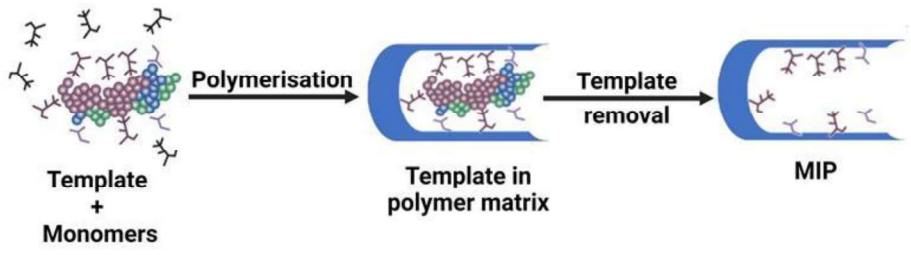

Figure 2: Creation and functionality of MIPs
Source: Own creation

The performance of MIPs depends on their molecular recognition capability, which is directly linked to their fabrication method. These polymers are designed to selectively bind their target molecules (templates) by exploiting structural compatibility (size and shape) and chemical affinity (functional groups). This selective binding occurs in a "lock-and-key" manner, similar to enzyme-substrate interactions. Typically, binding is mediated through non-covalent interactions such as hydrogen bonding, hydrophobic forces, or ionic interactions.

The binding of the target molecule to the receptor site in the MIP induces changes in the physical or chemical properties at the sensor surface. These changes are detected by transducers such as electrochemical, optical, or piezoelectric sensors, with electrochemical detection being the most widely used. MIP sensors exhibit high specificity, selectivity, and sensitivity, making them a promising platform for detecting drugs, pathogens, and other clinically relevant biomarkers. Their applications span the advanced diagnostics and therapeutic monitoring of conditions such as diabetes, hormonal imbalances, and inflammation, offering improved treatment outcomes and reduced healthcare costs (Li et al., 2024; Sajini & Mathew, 2021).

The aim of this research was to develop MIP sensors capable of monitoring cellular metabolites, such as insulin and lactate, in various in vitro tissue models.

2 Materials and methods

2.1 Materials

Pyrrole monomer (≥ 98 %), potassium ferrocyanide (K_3 [$Fe(CN)_6$]), potassium ferrocyanide (K_4 [$Fe(CN)_6$]), potassium chloride (KCl), and a standard lactate solution (1 g/l) were obtained from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS, 0,01 M, pH 7,2) was prepared according to the manufacturer's instructions by dissolving a PBS tablet (2005,5 mg) in 200 ml ultrapure water, following the manufacturer's instructions. A 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe was prepared in PBS (0,01 M, pH 7,2). Insulin solution was prepared by dissolving insulin in ultrapure water to achieve a final concentration of 0,58 mM. Lower-concentration insulin and lactate solutions were prepared by diluting the stock solutions in PBS (0,01 M, pH 7,2). All chemicals were of analytical grade and

used without further purification. Ultrapure water (resistivity: 18,2 MΩ·cm at 25°C) was obtained using the ELGA PureLab water purification system (Veolia Water Technologies, UK) and was used to prepare all solutions.

2.2 Instruments and electrodes

Electrochemical measurements were conducted using a PalmSens4 potentiostat/galvanostat (PalmSens, Houten, The Netherlands) at 23 ± 2°C. The PalmSens4 was controlled with PSTrace 5.8 software.

A screen-printed carbon electrode (SPCE), model AC1.W4.R2, supplied by BVT Technologies (Brno, Czech Republic) was used to prepare the MIP insulin sensor. A DropSens SPCE, model DRP11-L, supplied by Metronohm (Llanera, Spain) was used to prepare the MIP sensor for lactate.

Each SPCE system consisted of a three-electrode configuration with a carbon working electrode (WE), a carbon counter electrode (CE), and a silver/silver chloride (Ag/AgCl) reference electrode. All potentials reported in this study are referenced to the Ag/AgCl electrode.

2.3 Preparation of the MIP-electrode

The electrochemical preparation of an MIP sensor begins with the selection of an electrode for molecular imprinting of the target molecule. Various electrode types are available, including platinum, quartz crystal, nanocrystalline diamond, and graphite electrodes (Pilvenyte et al., 2023). In this study, screen printed carbon electrodes (SPCE) from two different manufacturers were used to prepare MIP sensors. Cyclic voltammetry (CV) was employed to monitor the formation of the MIP film on the SPCE surface and to evaluate the electrochemical activity of the prepared MIP. Square-wave voltammetry (SWV) was used to assess the rebinding of the analyte onto the MIP electrodes (Ozcelikay et al., 2019).

Before electrosynthesis of the MIP film, the electrochemical properties of the working electrode (WE), such as conductivity, were evaluated using CV and 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probes in PBS. As described in a previous study the formation of MIP films for insulin and lactate on the SPCE surface was performed via CV

(Zidaric et al., 2023). Pyrrole was chosen as the monomer due to its suitability for deposition on electrodes in aqueous solutions and its favorable properties prior to oxidation. These attributes allow the polypyrrole (Ppy) matrix to act as a synthetic antibody for the target molecule, which becomes imprinted in the polymer (Zidaric et al., 2023).

Electropolymerization was carried out by cycling the potential (E) between 0,000 V and 0,900 V, then back to 0,000 V, at a scan rate (ν) of 50 mV/s for 10 cycles, using a step potential (E_{step}) of 7,0 mV. During polymerization, template molecules (insulin or lactate) migrated toward the working electrode and were embedded in the forming Ppy film. After polymerization, the trapped template molecules were removed from the polymer matrix, leaving cavities complementary in both shape and functionality to the target molecule. A polymerization solution containing pyrrole (0,80 M) and insulin (0,58 mM) or lactate (1 g/L) in PBS (pH 7,2) at a monomer-to-template ratio of 1:4 was prepared. A 50 μL aliquot of the solution was applied to the SPCE, ensuring that all three electrodes were covered.

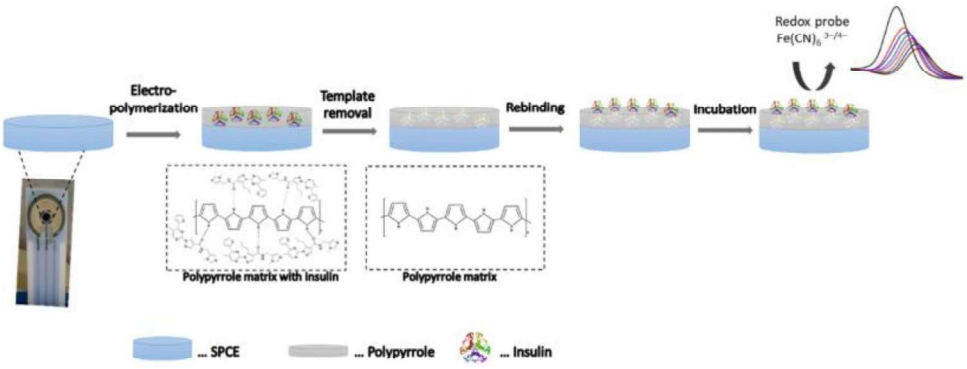

Figure 2: Schematic depiction of the steps involved in MIP sensor creation

Source: (Zidaric et al., 2023)

Following polymerization, electroelution was performed to remove the template molecules from the conducting polymer matrix. This step involved preoxidation of the Ppy matrix by cycling the potential between -0,200 V and 1,000 V for 25 cycles in PBS (0,01 M, pH 7,2) at a scan rate of 50 mV/s with an Estep of 7,0 mV. This process successfully prepared the electrode, making it suitable for the final step of development: rebinding the target molecule (insulin or lactate). At each step, the electrode was rinsed with ultrapure water and dried using compressed air.

2.4 Binding of the analyte to the prepared MIP-electrode

The electrochemical response of the MIP-electrode upon analyte rebinding was evaluated using SWV and CV. Measurements were performed with a 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe in 0,01 M PBS (pH 7,2).

For SWV, the potential was scanned in the anodic direction from -0,8 V to 0,6 V, with a frequency of 10 Hz, an amplitude of 100 mV, and an E_{step} of 10 mV. Voltammograms were recorded after applying a drop of the analytical solution to the MIP-electrode surface. Each measurement lasted approximately 25 seconds.

In addition, CV was employed to measure the electrochemical response of selected analytes. For insulin determination, the potential was scanned in the cathodic direction from 1,4 V to -0,6 V. For lactate determination, a potential range of -1,2 V to 0,7 V was used. In both analyses, a scan rate (ν) of 50 mV/s with an E_{step} of 7,0 mV was applied. Three replicate measurements were performed for each analyte to ensure reproducibility and reliability.

2.5 Reproducibility and Operator Bias

The experiments were conducted using several sets of SCPEs from multiple 75-count batches, noting the lot number, production date, and differences between manufacturers (DropSens and BVT Technologies). Reproducibility was evaluated within a batch and between batches using CV and redox probe $[Fe(CN)_6]^{3-/4-}$ (sweeping potential between -0,7 V and +0,7 V with a scan rate (ν) of 50 mV/s and an E_{step} of 7,0 mV). The results demonstrated high consistency and reliability.
Although handlers were not blinded to the experimental parameters, which introduces potential bias, all measurements were meticulously recorded and categorized and are available for further verification upon request.

3 Results and Discussion

3.1 Electrosynthesis (electropolymerization and electroelution) of MIP electrodes

The selection of appropriate support electrodes is a critical factor in the formation of MIP films on the working electrode surface. The electroanalytical performance of (bio)sensors is closely tied to the properties of the working electrode material, including electron transport, potential range, background current, and system stability. For this study, the goal was to develop a sensor compatible with miniaturized microphysiological systems (e.g., organs-on-a-chip). To meet this requirement, SPCEs were selected. These electrodes are increasingly employed in modern bioanalysis due to their advantages in microscale analysis (García-Miranda Ferrari et al., 2021). SPCEs are cost-effective, easy to use, and exhibit good reproducibility, making them ideal for bioanalytical applications.

In addition to these advantages, SPCEs allow efficient interaction with protein molecules without inducing denaturation, a common issue with metal electrodes such as gold, silver, and platinum (Contreras-Naranjo & Aguilar, 2019). Denaturation would negatively affect the electrode's ability to bind proteins, making SPCEs a superior choice for constructing sensors targeting biological molecules.

The quality and performance of SPCEs can vary depending on the batch and manufacturer. Variations in the formulation of the carbon paste, in the screen printing process or in the electrode production can lead to inconsistent results in electropolymerization. Therefore, before electrosynthesising MIPs, we evaluated the quality of electrical performance using CV and redox probe. On average, 10 electrodes per batch showed distorted measurements due to handling errors, calibration problems or manufacturing defects. The recognition and binding performance of synthetic MIP receptors depend heavily on the efficiency of the electropolymerization process. In this study, significant effort was devoted to optimizing the polymerization of pyrrole on the working electrode, as the electropolymerization conditions (e.g., potential range and number of cycles) directly influence the final electroanalytical performance of the MIP film. These parameters must be carefully tuned to create an optimal three-dimensional polymer structure. Excessive polymerization cycles can lead to overly thick and multilayered polymers,

hindering the analyte's ability to penetrate the polymer and reach the electrode surface. Additionally, too many cycles may result in layer delamination, compromising the polymer's structural integrity (Zidaric et al., 2023).

Template removal from the resulting polymer complex is another critical step in MIP electrosynthesis. Incomplete template removal can lead to false positive results during electrochemical measurements of the target analyte. Several template removal methods are available, including chemical (solvent-based), enzymatic, and electrochemical techniques. Each approach has its benefits and limitations. Based on prior research (Li & Qian, 2000; Zidaric et al., 2023), electroelution was chosen as the most effective method for template removal while maintaining the polymer's integrity.

Electroelution involves a broader voltage range and more cycles than polymerization, allowing for better polymer chain opening and thorough template removal (Zidaric et al., 2023). This process relies on the preoxidation of Ppy in aqueous solutions like PBS, which promotes the formation of oxygen-containing compounds during water oxidation. Carboxyl groups form on the pyrrole rings, leading to chain scission, which facilitates the removal of template molecules. The resulting imprinted cavities are functionalized for selective re-binding of target molecules through interactions between the analyte's functional groups and the carboxyl groups within the cavities (Li & Qian, 2000; Ratautaite et al., 2021; Zidaric et al., 2023).

This optimized electrosynthesis protocol, involving precise polymerization conditions and effective template removal through electroelution, ensures the formation of high-quality MIP films. These films are capable of selectively rebinding target molecules, thus enhancing the sensor's analytical performance and suitability for applications in microphysiological systems.

3.2 Re-binding of the target molecule to the insulin MIP-electrode

The binding of insulin to the polymer matrix gaps was primarily monitored using SWV, a highly sensitive and rapid voltametric method. SWV offers a detection limit comparable to spectroscopic and chromatographic techniques (Simões & Xavier, 2017). SWV is more effective than CV in reducing measurement noise, making it

ideal for accurate quantification of electric current during potential changes (Mirceski et al., 2018).

After template removal, the insulin MIP-electrode's performance was evaluated by applying a single drop of insulin solution and measuring the electrochemical response. Insulin concentrations ranging from 20,0 to 70,0 pM were tested by applying 5 μL of insulin solution to the sensor and incubating it for 15 minutes to allow binding to the polymer matrix. A redox probe, $[Fe(CN)_6]^{3-/4-}$, was then added, and the response was measured via SWV. While longer incubation times may reduce the detection limit, they also increase the risk of non-specific binding, which can compromise sensor selectivity.

Sequential testing of insulin solutions at increasing concentrations revealed a decrease in the redox probe signal after the first incubation (Figure 3). This decrease is attributed to insulin re-binding to the imprinted recognition sites on the MIP-electrode surface. The binding of insulin interferes with the diffusion of the redox probe to the working electrode surface, causing a signal reduction. This effect was observed to intensify with increasing insulin concentration.

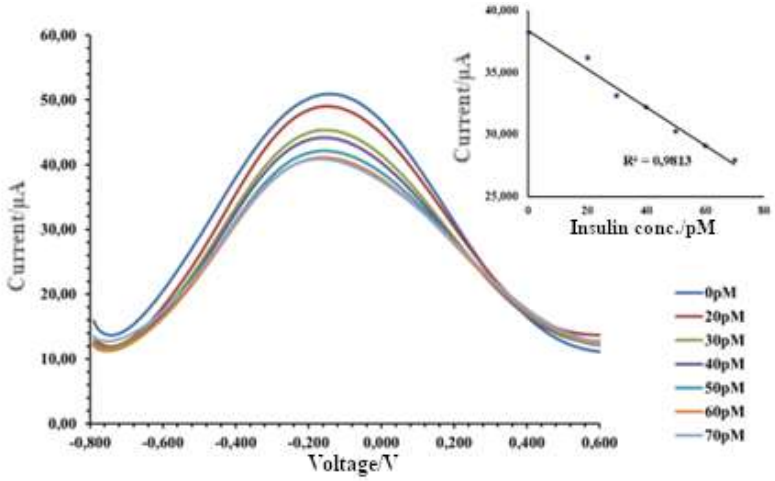

Figure 3: SWV voltammogram of insulin solution on the MIP electrode with an associated calibration curve (inset) over the concentration range of 0 to 70 pM

Source: Own creation

One of the challenges in using SPCEs is achieving uniform and controlled surface modifications. Variability between batches and manufacturers can lead to differences in the formulation of the carbon paste, the screen-printing process, or the electrode fabrication, all of which influence the polymerization process and the electrochemical properties of the MIP film. These inconsistencies may result in variability in SPCE properties, such as surface roughness, porosity, or impurity levels, which in turn affect the reliability and reproducibility of experimental data (Martindale et al., 2011; Suresh et al., 2021).

For highly sensitive methods like SWV, such variability can produce inconsistent results, including non-uniform electrochemical activity and poor signal-to-noise ratios. This leads to distorted voltametric signals and reduced measurement sensitivity and accuracy. In comparison, CV typically offers a more stable background signal, especially when measurements are influenced by high-frequency noise or low currents (Martindale et al., 2011). CV also provides better-defined voltametric signals and clearer resolution of redox peaks.

To address these issues, CV measurements were performed on MIP electrodes for insulin determinations. The parameters were adjusted to achieve an optimal current response and well-resolved peaks. It was also observed that the direction of the CV measurement (starting from the cathode or anode) influenced the noise level. Measurements initiated in the anodic direction were more susceptible to noise due to solvent oxidation, which generates background current unrelated to the analyte (Yamada et al., 2022). Consequently, measurement parameters were modified to begin in the cathodic direction (from positive to negative voltage) over a range of 1,4 V to -0,6 V, which reduced noise and improved signal clarity.

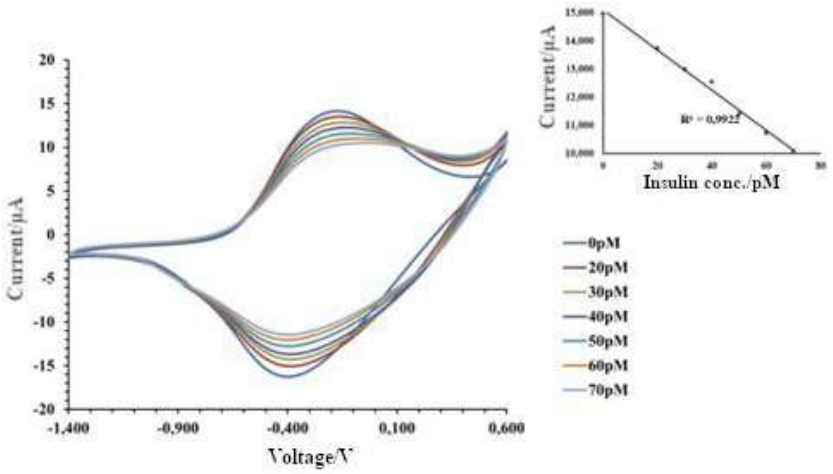

Figure 4: CV analysis of insulin re-binding to the MIP-electrode with an associated calibration curve (inset) over the concentration range of 0 to 70 pM

Source: Own creation

As shown in Figure 4, the signal decreased linearly with increasing insulin concentrations, reflecting insulin binding to complementary cavities in the MIP film. CV measurements were less sensitive to batch-to-batch variations in SPCEs compared to SWV. Although SWV offers higher resolution, its sensitivity to electrode variability limited its advantage in this case. CV provided more consistent results, suggesting that it may be more reliable for insulin determination under these experimental conditions (Martindale et al., 2011).

After removal of the template, the performance of MIP-SPCE was evaluated using the SWV technique in a single drop of insulin solution (as described above). The relationship between the insulin concentration and SWV signal was investigated in the range of 20,0 to 70,0 pM. For this purpose, 5 µl of insulin solutions of different concentrations were incubated for 15 minutes to facilitate insulin adsorption prior to application of the $[Fe(CN)_6]^{3-/4-}$ redox probe and subsequent measurement of the electrochemical response by SWV. After the first insulin incubation, a significant decrease in the Δi_p of the redox probe was observed (Figure 3). This decrease was attributed to the rebinding of insulin to the imprinted recognition sites on the surface of the MIP film, with the effect becoming more pronounced with increasing insulin concentration. The data showed a clear linear relationship between Δi_p and insulin concentration in a concentration range of 20,0 to 70,0 pM (see Figure 3). Compared

to previous insulin detection methods (Table 1), the MIP-SPCE sensor achieved comparable or lower limits of detection (LOD). However, a key advantage of this approach is its ability to analyze insulin with a low LOD using only a single drop of analyte sample, which distinguishes it from other methods.

3.3 Re-binding of the target molecule to the lactate MIP-electrode

This study assessed the ability of a lactate-specific MIP-electrode to quantify lactate concentrations within the physiological range for healthy tissues (1,5 to 3 mM) (Li et al., 2022). The protocol for re-binding lactate to the synthesized receptor sites on the MIP-electrode was adapted and optimized (Figure 5). Electrochemical measurements were performed within a voltage range of -1,2 V to 0,7 V using CV and the redox probe $[Fe(CN)_6]^{3-/4-}$.

o facilitate binding, 5 µL of lactate solution at varying concentrations was applied to the MIP-electrode and incubated for 10 minutes. The results revealed a decrease in current response as lactate concentration increased. This signal reduction is attributed to the binding of lactate molecules to the MIP film, which interferes with potassium ion (K^+) transfer and the associated electron exchange at the working electrode surface (Dykstra et al., 2024). These results confirm the MIP-electrode's functionality and its ability to detect lactate within the targeted concentration range.

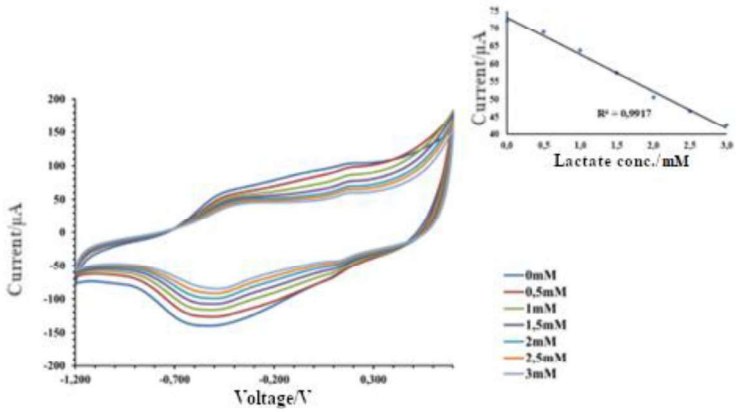

Figure 5: Current response of lactate re-binding to the MIP-electrode with an associated calibration curve (inset) over the concentration range of 0 to 3 mM

Source: Own creation

To evaluate the specificity of lactate recognition, a non-molecularly imprinted polymer (NIP) film was synthesized and tested under the same conditions. Unlike the MIP, the NIP film was electrosynthesized in the absence of lactate, resulting in a polymer matrix lacking complementary recognition sites. Any interaction between lactate and the NIP film would therefore arise from non-specific binding to the Ppy matrix.

The results from the NIP-electrode demonstrated a non-linear response to increasing lactate concentrations within the same range where the MIP-electrode exhibited a linear response (Figure 6). This non-linear behavior highlights the specificity of the receptor sites in the MIP film, confirming that the observed responses in the MIP-electrode were primarily due to specific lactate binding rather than non-specific interactions.

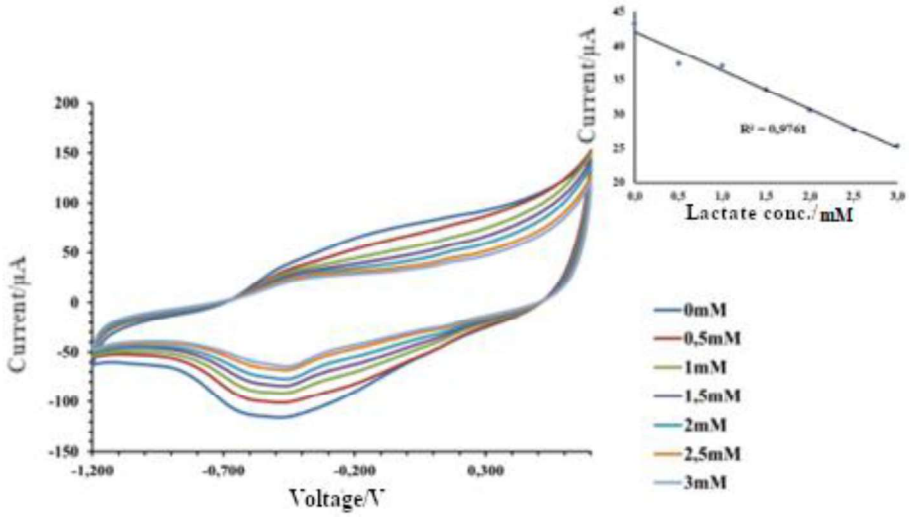

Figure 6: Response of increasing lactate concentrations to NIP electrodes with an associated calibration curve (inset) over the concentration range of 0 to 3 mM

Source: Own creation

This analysis underscores the effectiveness of the MIP-electrode in selectively binding and quantifying lactate, validating its potential application in physiological monitoring systems. The comparison with NIP-electrodes further demonstrates the high specificity of the MIP film, supporting its utility for precise lactate detection.

3.4 Results

The results presented in this article regarding rebinding to the lactate MIP electrode are preliminary, as the tests have not yet been finalized, including determining LOD and LOQ values. At this stage, we aim to demonstrate the feasibility of creating specific receptor targets for small molecules such as lactate using MIP development methods.

Table 1: A comparison of the linear concentration ranges, LODs, and real sample applications of different electrochemical sensors for insulin detection published previously.
Source: 1: (Salimi et al., 2008) 2: (Prasad et al., 2010) 3: (Martínez-Periñán et al., 2016) 4: (Guo et al., 2012) 5: (Gerasimov et al., 2013) 6: (Ebrahimiasl et al., 2018) 7. (Singh, 2019) 8. (Shepa et al., 2021) 9. Our research

Sensor	Technique	Linear concentration range	LOD	Real sample	Source
Guanine/NiO_x/GCE	Amperometry	100 pM-4μM	22 pM	N/A	1
MWCNT/MIP	DPASV	0.068-5.682 nM	0,0183 nM	Blood serum, insulin injection	2
$Ni(OH)_2$NPs/Nafion-MWCNTs/GCE	CV	1.5-40 μM	85 nM	Human plasma, pharmaceuticals	3
AB/CPE	DPV	20-1000 nM	5 nM	Insulin injection	4
Aptamer (In1-IT)-based sensor	ACV	10-200 nM	10 nM	N/A	5
Ppy-GF/PGE	CA	0.225-1.235 μM	8,65 nM	Blood serum	6
SPE/MWNT-QD	SWV	100-5000 pM	100 pM	N/A	7
SPCE/MWCNT/$NiO_{1.5}$	Amperometry	600 nM-10 μM	19,6 nM	Blood serum	8
MIP-SPCE	SWV	20.0-70.0 pM	2,14 pM	N/A	9

4 Conclusion

This study demonstrates the ability of MIP electrodes to analyze small sample volumes of insulin and lactate in physiological concentrations (insulin: 20,0 – 70,0 pM; lactate: 1,5-3,0 mM) without requiring additional nanomaterials or complex immobilization compounds (e.g., enzymes, antibodies, or aptamers). This can reduce both production costs and preparation time. The system's miniaturization and rapid response time offer a significant advantage over traditional biochemical methods. Additionally, the potential integration of MIP sensors into portable devices makes

them ideal candidates for point-of-care applications, representing a step forward in accessible and efficient diagnostic technologies.

This research aimed to demonstrate the applicability of MIP sensors for detecting single analytes in clinical practice and among individuals under high physical stress, such as professional athletes and soldiers. Wearable devices incorporating MIP technology could enable continuous, real-time physiological status monitoring, revolutionizing personal health and sports science. These devices would precisely track physiological processes, optimizing performance and health management (Ayankojo et al., 2024).

In addition, MIP sensors show great potential in diabetes management by enabling early detection of insulin level deviations and ensuring timely treatment adjustments. Additionally, lactate monitoring with these sensors aids in identifying metabolic changes, which are crucial in intensive care, emergency medicine, and cardiovascular care (Psoma & Kanthou, 2023).

Conventional analytical methods require manual sampling, large working volumes, and frequent system interruptions, making them unsuitable for miniaturized systems such as Transwell inserts, organ-on-a-chip models, and organoids. These methods often rely on optical and fluorescence microscopy with dyes and labels, which only allow single measurements and often require experiment termination. In contrast, miniaturized MIP-based sensors allow convenient integration into various in vitro models and in situ monitoring without negatively affecting the cells. In addition, synthetic recognition units are more stable to external factors (e.g., pH, temperature) than natural ones (e.g., enzymes, antibodies). The electrosynthesized MIP receptors also allow precise control of polymer film thickness, increasing biorecognition sensitivity.

Despite the promising results, some limitations have appeared during our study. One of the key challenges is the variability of the sensor performance, mainly due to the fabrication of the screen-printed carbon electrode (SPCE), which highlights the need for further optimization to improve the sensor's reliability. In addition, our study does not include a direct comparison of sensor performance with established clinical methods such as those used in clinical practice. However, we believe that MIP sensors could significantly improve the accuracy, stability and functionality of clinical

sensors such as the FreeStyle Libre. By offering a non-enzymatic, highly specific and cost-effective alternative, they promise to improve patient outcomes and reduce healthcare costs. For example, the FreeStyle Libre relies on enzymatic glucose sensors, which can be interfered with by other substances in the blood or interstitial fluid (e.g. paracetamol, uric acid). MIP sensors could reduce such cross-reactivity as recognition is based on the shape in functionally orientated imprinted cavities that resemble the template molecule. Due to the stable polymer matrix, they could have a longer lifetime so that the sensors need to be replaced less frequently. In addition, MIP sensors could enable simultaneous monitoring of multiple analytes (e.g. glucose, lactate and cortisol), providing a more comprehensive overview of the patient's metabolic status in a single device.

Future research should focus on the applicability of MIP sensors for detecting other analytes and wider concentration ranges of these analytes. Studies will also be needed to establish their effectiveness and suitability for use in clinical practice. Addressing these challenges will not only improve the functionality of MIP sensors but will also allow their integration into advanced diagnostic technologies.

Notes

Abbreviations used in the text:

OOC: organ-on-a-chip
MIP: molecularly imprinted polymer
SPCE: screen printed carbon electrode
CV: cyclic voltammetry
SWV: square wave voltammetry
Ppy: polypyrrole
NIP: non-molecularly imprinted polymer
POC: point of care

Acknowledgments

The authors acknowledge the financial support from the Slovenian Research and Innovation Agency for Research Core Funding No. P3-0036, and through projects No. L7-4494, N1-0305, and J3-4524.

Literature

Ayankojo, A. G., Reut, J., & Syritski, V. (2024). Electrochemically Synthesized MIP Sensors: Applications in Healthcare Diagnostics. *Biosensors (Basel)*, *14*(2), 71. https://doi.org/10.3390/bios14020071

Contreras-Naranjo, J. E., & Aguilar, O. (2019). Suppressing Non-Specific Binding of Proteins onto Electrode Surfaces in the Development of Electrochemical Immunosensors. *Biosensors (Basel)*, *9*(1). https://doi.org/10.3390/bios9010015

Dykstra, G., Chapa, I., & Liu, Y. (2024). Reagent-Free Lactate Detection Using Prussian Blue and Electropolymerized-Molecularly Imprinted Polymers-Based Electrochemical Biosensors. *ACS Appl Mater Interfaces*, *16*(49), 66921-66931. https://doi.org/10.1021/acsami.3c19448

Ebrahimiasl, S., Fathi, E., & Ahmad, M. (2018). Electrochemical Detection of Insulin in Blood serum using Ppy/GF Nanocomposite Modified Pencil Graphite Electrode. *Nanomedicine Research Journal*, *3*(4). https://doi.org/10.22034/nmrj.2018.04.006

García-Miranda Ferrari, A., Rowley-Neale, S. J., & Banks, C. E. (2021). Screen-printed electrodes: Transitioning the laboratory in-to-the field. *Talanta Open*, *3*. https://doi.org/10.1016/j.talo.2021.100032

Gerasimov, J. Y., Schaefer, C. S., Yang, W., Grout, R. L., & Lai, R. Y. (2013). Development of an electrochemical insulin sensor based on the insulin-linked polymorphic region. *Biosens Bioelectron*, *42*, 62-68. https://doi.org/10.1016/j.bios.2012.10.046

Guo, C., Chen, C., Luo, Z., & Chen, L. (2012). Electrochemical behavior and analytical detection of insulin on pretreated nanocarbon black electrode surface. *Analytical Methods*, *4*(5). https://doi.org/10.1039/c2ay05828f

Kumar, D., Nadda, R., & Repaka, R. (2024). Advances and challenges in organ-on-chip technology: toward mimicking human physiology and disease in vitro. *Med Biol Eng Comput*, *62*(7), 1925-1957. https://doi.org/10.1007/s11517-024-03062-7

Li, X., Yang, Y., Zhang, B., Lin, X., Fu, X., An, Y., Zou, Y., Wang, J. X., Wang, Z., & Yu, T. (2022). Lactate metabolism in human health and disease. *Signal Transduct Target Ther*, *7*(1), 305. https://doi.org/10.1038/s41392-022-01151-3

Li, Y., Luo, L., Kong, Y., Li, Y., Wang, Q., Wang, M., Li, Y., Davenport, A., & Li, B. (2024). Recent advances in molecularly imprinted polymer-based electrochemical sensors. *Biosens Bioelectron*, *249*, 116018. https://doi.org/10.1016/j.bios.2024.116018

Li, Y., & Qian, R. (2000). Electrochemical overoxidation of conducting polypyrrole nitrate film in aqueous solutions. *Electrochimica Acta*, *45*(11), 1727-1731. https://doi.org/10.1016/s0013-4686(99)00392-8

Martindale, B. C., Aldous, L., Rees, N. V., & Compton, R. G. (2011). Towards the electrochemical quantification of the strength of garlic. *Analyst*, *136*(1), 128-133. https://doi.org/10.1039/c0an00706d

Martínez-Periñán, E., Revenga-Parra, M., Gennari, M., Pariente, F., Mas-Ballesté, R., Zamora, F., & Lorenzo, E. (2016). Insulin sensor based on nanoparticle-decorated multiwalled carbon nanotubes modified electrodes. *Sensors and Actuators B: Chemical*, *222*, 331-338. https://doi.org/10.1016/j.snb.2015.08.033

Mirceski, V., Skrzypek, S., & Stojanov, L. (2018). Square-wave voltammetry. *ChemTexts*, *4*(4), 17. https://doi.org/10.1007/s40828-018-0073-0

Ozcelikay, G., Kurbanoglu, S., Zhang, X., Kosak Soz, C., Wollenberger, U., Ozkan, S. A., Yarman, A., & Scheller, F. W. (2019). Electrochemical MIP Sensor for Butyrylcholinesterase. *Polymers (Basel)*, *11*(12), 1970. https://doi.org/10.3390/polym11121970

Pilvenyte, G., Ratautaite, V., Boguzaite, R., Ramanavicius, S., Chen, C. F., Viter, R., & Ramanavicius, A. (2023). Molecularly Imprinted Polymer-Based Electrochemical Sensors for the Diagnosis of Infectious Diseases. *Biosensors (Basel)*, *13*(6), 620. https://doi.org/10.3390/bios13060620

Prasad, B. B., Madhuri, R., Tiwari, M. P., & Sharma, P. S. (2010). Imprinting molecular recognition sites on multiwalled carbon nanotubes surface for electrochemical detection of insulin in real samples. *Electrochimica Acta*, *55*(28), 9146-9156. https://doi.org/10.1016/j.electacta.2010.09.008

Psoma, S. D., & Kanthou, C. (2023). Wearable Insulin Biosensors for Diabetes Management: Advances and Challenges. *Biosensors (Basel)*, *13*(7), 719. https://doi.org/10.3390/bios13070719

Ratautaite, V., Samukaite-Bubniene, U., Plausinaitis, D., Boguzaite, R., Balciunas, D., Ramanaviciene, A., Neunert, G., & Ramanavicius, A. (2021). Molecular Imprinting Technology for Determination of Uric Acid. *Int J Mol Sci*, *22*(9). https://doi.org/10.3390/ijms22095032

Sajini, T., & Mathew, B. (2021). A brief overview of molecularly imprinted polymers: Highlighting computational design, nano and photo-responsive imprinting. *Talanta Open*, *4*, 100072. https://doi.org/10.1016/j.talo.2021.100072

Salimi, A., Noorbakhash, A., Sharifi, E., & Semnani, A. (2008). Highly sensitive sensor for picomolar detection of insulin at physiological pH, using GC electrode modified with guanine and electrodeposited nickel oxide nanoparticles. *Biosens Bioelectron*, *24*(4), 798-804. https://doi.org/10.1016/j.bios.2008.06.046

Shepa, J., Sisolakova, I., Vojtko, M., Trnkova, L., Nagy, G., Maskalova, I., Orinak, A., & Orinakova, R. (2021). NiO Nanoparticles for Electrochemical Insulin Detection. *Sensors (Basel)*, *21*(15). https://doi.org/10.3390/s21155063

Simões, F. R., & Xavier, M. G. (2017). Electrochemical Sensors. In A. L. Da Róz, M. Ferreira, F. de Lima Leite, & O. N. Oliveira (Eds.), *Nanoscience and its Applications* (pp. 155-178). William Andrew Publishing. https://doi.org/10.1016/b978-0-323-49780-0.00006-5

Singh, V. (2019). Quantum dot decorated multi-walled carbon nanotube modified electrochemical sensor array for single drop insulin detection. *Materials Letters*, *254*, 415-418. https://doi.org/10.1016/j.matlet.2019.07.122

Suresh, R. R., Lakshmanakumar, M., Arockia Jayalatha, J. B. B., Rajan, K. S., Sethuraman, S., Krishnan, U. M., & Rayappan, J. B. B. (2021). Fabrication of screen-printed electrodes: opportunities and challenges. *Journal of Materials Science*, *56*(15), 8951-9006. https://doi.org/10.1007/s10853-020-05499-1

Yamada, H., Yoshii, K., Asahi, M., Chiku, M., & Kitazumi, Y. (2022). Cyclic Voltammetry Part 1: Fundamentals. *Electrochemistry*, *90*(10), 102005-102005. https://doi.org/10.5796/electrochemistry.22-66082

Zidaric, T., Majer, D., Maver, T., Finsgar, M., & Maver, U. (2023). The development of an electropolymerized, molecularly imprinted polymer (MIP) sensor for insulin determination using single-drop analysis. *Analyst*, *148*(5), 1102-1115. https://doi.org/10.1039/d2an02025d