Sensors & Actuators: B. Chemical 376 (2023) 132928

Contents lists available at ScienceDirect

Sensors and Actuators: B. Chemical

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

Smart nano-actuators for electrochemical sensing of Metformin in human plasma

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ARTICLE INFO

Keywords:
Electrochemical sensor
Molecularly imprinted polymer nanoparticles
Metformin
Nano-actuator

ABSTRACT

Smart artificial nano-actuators were developed using electroactive acrylamide polymer nanoparticles by molecular imprinting. These nanoparticles are receptors that mimic the specificity of antibodies and replace enzymes in traditional biosensors allowing highly sensitive and selective monitoring. Electroactive polymer nanoparticles can be synthesized by the including of ferrocene, which confers signaling functions. These actuators operate by a swelling mechanism in response to a specific electrochemical stimulus. Molecular recognition is based on molecular imprinting and binding of the target molecule. The polymer nanoparticles were computationally designed to bind to Metformin and synthesized by controlled free-radical polymerization. Polymer nanoparticles were incorporated on the surface using screen-printed electrodes. As a result, these nano-actuators allow extremely sensitive monitoring of Metformin in human plasma. Metformin is essential for treating various chronic diseases, and monitoring is necessary to reduce collateral drug side effects. Herein, sensing of Metformin was achieved by differential pulse voltammetry (DPV) in a concentration range between 100 and 2000 pM, with a limit of detection of 9 pM and sensitivity at 31.5 nA pM^{-1} (0.998) in plasma. No cross-reactivity was observed against potential interferences (Sitagliptin and Paracetamol). This technology can be potentially applied for rapid point-of-care testing as an alternative to laboratory-based techniques, reducing the time and cost of the analysis.

1. Introduction

Metformin is well-known because of the pleiotropic effect and the wide range of therapeutic effects in several chronic diseases. [1] It is used primarily to treat diabetes (DMT2), but it also has the potential for use in cardiovascular medicine, neurodegenerative diseases, cancer and preventing DMT2. [2–6] Other factors, such as its low price and minimal side effects, decrease mortality rate and contribute to the value of Metformin as an oral anti-diabetic drug. [7] However, dosage monitoring is critical to avoid long-term secondary effects. [8] For example, in patients with kidney problems, Metformin can be accumulated in the blood, causing lactic acidosis. [9,10] Evidence suggests that excessive Metformin administration may contribute to poor renal function, as well as anemia, vitamin B12 deficiency, and gastrointestinal issues. [11–13] Due to the high rates of prescription and consumption, dosage monitoring is, therefore, of great importance.

Metformin is typically administrated orally (40–60% bioavailability), [14] and Metformin is absorbed in the upper small intestine. [15,16] It is not metabolized, thus eliminated unchanged in the urine.[14] The rate of elimination is faster than that of absorption. [14] Therefore, the therapeutic Metformin plasma concentration range depends on the disease treated and the bioequivalence protocols employed. Minimal active plasma concentrations are approximately 0.225 μg L^{-1} (1.74 nM) and should not exceed 5 mg L^{-1} (39 μM), which is the toxic threshold.[1,17] Surprisingly, the relationship between dose-efficacy and plasma Metformin concentrations is still unclear. [1] Thus, it is challenging to determine dosage levels, accumulation, and prognosis. [18]Therefore, there is a need for an efficient and rapid test to track Metformin plasma levels.

They are several analytical methods for Metformin quantification employed in the analysis of complex biological samples, as shown in Table S1. [19,20] The most common techniques include: calorimetry, [21] spectroscopy, [22] spectrofluorimetry, [23] high-performance liquid chromatography (HPLC), [24] liquid chromatography coupled

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https://doi.org/10.1016/j.snb.2022.132928
Received 9 June 2022; Received in revised form 27 October 2022; Accepted 29 October 2022
Available online 2 November 2022

with mass spectrometry (LC-MS/MS), [25] reverse-phase liquid chromatography (RP-LC), [26] and capillary electrophoresis coupled with electro- chemiluminescence (CE-ECL).[27] Overall, these analytical methods offer adequate sensitivity and low detection limits. Nevertheless, they require a comprehensive analysis by qualified personnel, in addition to tedious and lengthy sample preparation steps, which make them unsuitable for regular monitoring and point-of-care diagnostics. Therefore, portable, sensitive, highly selective sensors capable of detecting low concentrations of Metformin in complex mixtures such as human plasma are highly desirable.[28].

Biosensors and chemical sensors offer rapid measurement and response, user-friendly platforms, portability, low-cost manufacturing, and high sensitivity. [29,30] The global market for disposable sensors has increased tremendously, especially for medical diagnostics, food monitoring, and environmental applications. [29,31] Usually, chemical sensors are based on the electrochemical oxidation of the analyte, which may lead to interferences, poor selectivity, and applicability in biological samples. Alternatively, biosensors commonly use natural receptors such as antibodies, enzymes, and aptamers to detect specific targets. [32] Unfortunately, these biological components are susceptible to moisture, temperature, and pH variations and are denatured in harsh conditions. As an alternative, molecularly imprinted polymers (MIPs) have been used as synthetic receptors in sensors, usually as coatings or microbeads. [33,34].

MIPs provide the high sensitivity and selectivity of biological receptors, while their polymeric nature makes them resistant to harsh conditions. [31,35] However, challenges may be presented when applied to biological samples, including cross-reactivity. [36] More recently, molecularly imprinted nanoparticles (NanoMIPs) were employed as replacements for enzymes and antibodies in assays and sensors, presenting extraordinary sensitivity and selectivity in biological samples.[35,37,38] The use of NanoMIPs allows producing the uniform recognition cavities, resulting in an enhanced specificity and overall performance over traditional techniques.[37,39] The regular distribution of analyte-binding sites also results in efficient mass transfer leading to rapid analyte recognition. [37,40] Moreover, NanoMIPs are easy to synthesize and integrate into a variety of transducers including electrochemical devices. [38–40].

This work presents the design, synthesis, and integration of smart, stimuli-responsive nanomaterials onto screen-printed platinum electrodes (SPPE). These nano-actuators were prepared using electroactive acrylamide polymer nanoparticles, prepared using molecularly imprinting. The electroactivity of the particles is imparted by introducing a redox marker (ferrocene) into the polymer system, which confers signaling properties.[38] Moreover, the particles are specific due to the recognition cavities designed and optimized *in silico using* monomer screening.[37] The monomer composition and the parameters used during the solid-phase synthesis controlled the size and properties. [35,38] The resulting Metformin sensor exhibited excellent sensitivity in spiked human plasma samples while maintaining high stability and performance.

Herein, nanoparticles are used as sensors. These nanoparticles respond to a physical stimulus and transmit a resulting impulse into electrical signals.[32] The mechanism behind this process is particle actuation.[41] The analyte recognition process triggers a polymer conformation change, generating an electric signal. Therefore, these nanoparticles are actuators; they produce a molecular motion by recognizing the analyte. The Nano actuation modifies the electroactivity of the ferrocene present in the particles. Consequently, the motion of the nanoparticles is converted into an electric signal. This actuation mechanism is known as “Induced fit“ in enzymes.[42,43] The actuation mechanism was experimentally evidenced by measuring the diameter of nanoparticles in water using DLS.[38] The diameter of NanoMIPs increases size at 23% in the presence of the target analyte. Furthermore, NanoMIP actuation is specific to the target since no changes were observed in the presence of other molecules. Thus, these Nano-actuators mimic the specificity of antibodies and replace enzymes in traditional biosensors allowing highly sensitive Metformin monitoring in human plasma.

2. Material and method

Glass microbeads (GB) SPHERIGLASS® A-Glass 2429 (106 – 150 μm diameter) were obtained from Potters Industries LLC. Ethylene glycol methacrylate phosphate (EGMP) was acquired from Alfa-Chemistry USA. Pentaerythritol tetrakis(3-mercaptopropionate) (PETMP), *N,N′*-methylene-bis-acrylamide (MBA), trimethylolpropane trimethacrylate (TRIM), *N*-(3- aminopropyl)methacrylamide hydrochloride (NAPMA), 1,2-Bis(triethoxysilyl)ethane (BTSE), ferrocenylmethyl methacrylate (FcMMA), ethanolamine, glutaraldehyde, 2- (trifluoromethyl)acrylic acid (TFMAA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), sodium cyanoborohydride, *N,N*- diethyldithiocarbamic acid benzyl ester (iniferter), 3-aminopropyl)triethoxysilane (APTES), 5- (dimethylamino)naphthalene-1-sulfonyl chloride (DNSCl), phosphate buffered saline (PBS), itaconic acid (ITA), dodecylamine (DDA), ninhydrin, acetonitrile, dimethylformamide (DMF), ethanol, toluene, sodium hydroxide (NaOH), Metformin and Sitagliptin phosphate monohydride were purchased from Sigma-Aldrich, UK. Double-distilled ultrapure water (Millipore, UK) was used for all experiments. All solvents and chemicals were of HPLC or analytical grade and treated without purification.

3. Instrumentation and measurements

Metformin imprinted nano-actuators were designed using the molecular modeling of the optimal analyte geometries and functional monomer screening using the software package Sybyl 7.3 (Tripos Inc., USA) in a Gnome 2.28.2 (Linux) environment. Molecular modeling was carried out using a HP Elite-Desk with two Intel Core™ Duo CPU E8400 and 3 GHz processors running on a CentOS Linux 7 operating system. Polymerization of NanoMIPs was performed using a UV-lamp (0.5 W cm^{-1}, 4 × 15 W, Philips HB171/A, Germany). Solid phase extraction cartridges (SPE) with polyethylene frits (20 μm porosity, Supelco, UK) were used for the elution of NanoMIPs. Purification was performed by filtration and dialysis using disposable plastic syringes with cellulose acetate filters (25 mm, 0.45 μm, Whatman) and SnakeSkin dialysis tubes (Spectra / Por 7, regenerated cellulose, reference 132119, 10 kDa MWCO, tube length 11 cm, flat width 32 mm, diameter 20.4 mm, 3.3 mL cm^{-1}). Dynamic light scattering (DLS) was employed to measure the hydrodynamic diameter of the particles using a Zetasizer Nano (Malvern Instruments Ltd, UK). For these measurements, 500 μL solution of nanoparticles in water at 25 °C was previously sonicated for 1 min using an Ultrasonic bath (Hilsonic Ultrasonic Cleaner, 20–100 kHz, 220 V).

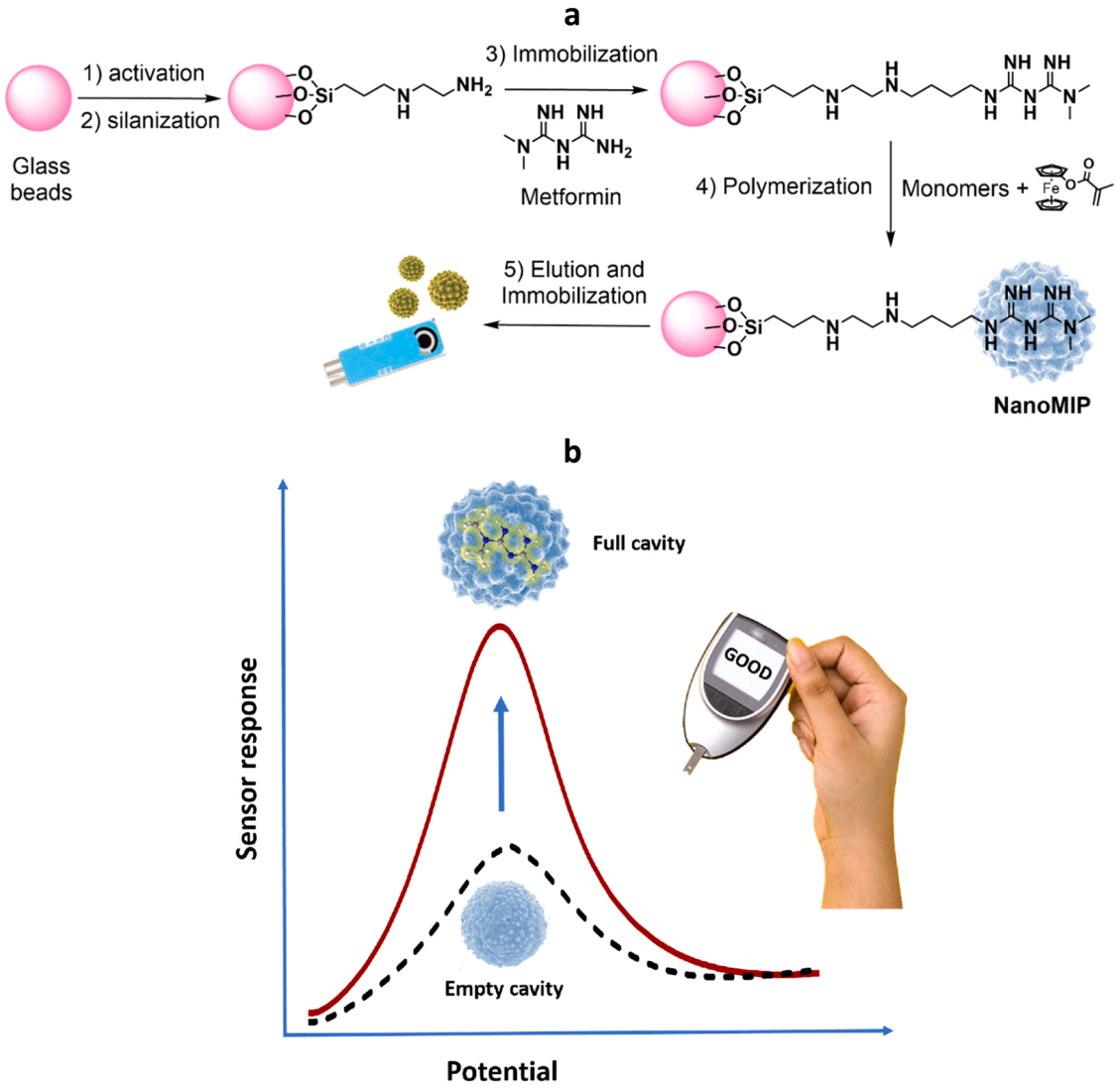
The chemical structure was confirmed using the infrared spectroscopy analysis using an ATR-FTIR spectrometer (Bruker Alpha Platinum). Transmission electron microscopy (TEM) images were obtained in a JEOL JEM-1400 TEM with an accelerating voltage of 120 kV, equipped with EMSIS Xarosa digital camera with Radius software. Samples were sonicated for 2 min immediately prior to adsorption to the grid. 10 μL of sample were then applied to a freshly glow discharged carbon film grid (400 mesh, AGS160-Agar Scientific Ltd). Grids were glow discharged in a Quorum GloQube System for 15 s at 20 mA. The sample was then allowed to adsorb and dry for 25 min under ambient conditions.at room temperature. The electrochemical measurements were performed using screen-printed platinum electrodes (SPPE, Dropsens DRP-550, 3.4 ×1.0 ×0.05 cm) purchased from Metrohm, UK. The electrode surface was cleaned and activated by using a plasma cleaner instrument (Emitech, K1050X RF Plasma Cleaner, 50 W, 13.56 MHz RF for 5 min). All the electrochemical measurements were performed by using a Potentiostat (PalmSens4, Netherlands) and data acquisition was operated with a PSTrace 5 software (PalmSens, Netherlands).

3.1. Synthesis of Metformin-imprinted nano-actuators

The solid phase was first activated and silanized as shown in Fig. S1. The Metformin was then covalently immobilized on silanized glass microspheres (Met-GB) as shown in Fig. S2 (detailed information is provided in the supplementary information, Section 2). The Metformin-imprinted polymer nanoparticles were synthesized by Reversible Addition– Fragmentation chain Transfer (RAFT) using photo-polymerization in the presence of the glass beads with immobilized Metformin (Met-GB). The NanoMIPs polymerization mixture comprised following: FcMMA (0.17 g, 27.1 mmol) as redox marker, Iniferter (0.75 g, 3.14 mmol), PETMP (0.18 g, 0.37 mmol) as chain-transfer agent, MBA (2.52 g, 16.35 mmol), NAPMA (0.02 g, 0.17 mmol) and TRIM (3.24 g, 9.57 mmol) as cross-linkers. EGMP (7.03 g, 33.47 mmol), ITA (4.35 g, 33.47 mmol) and TFMAA (2.50 g, 17.85 mmol) were selected as functional monomers using computational chemistry. All polymerization components were dissolved in 25 mL of acetonitrile and 30 g of Met-GB were added. Then, the polymerization mixture was then degassed for 15 min with nitrogen and sonicated for 3 min. To complete the polymerization, the mixture was exposed to UV radiation for 90 s. Following polymerization, the supernatant was removed and the solid phase was transferred to the SPE cartridge for the elution process, as shown in Scheme 1. The solid phase was washed with cold acetonitrile (3 × 120 mL at 0 °C) to eliminate residues, followed by NanoMIPs elution from the solid phase by washing (5 × 20 mL at 60 °C) with ethanol. The eluted NanoMIPs (100 mL in ethanol) were concentrated to 10 mL. For the purification, NanoMIPs (2 mL) was diluted in distilled water (8 mL). The purification was performed using a dialysis membrane (MWCO: 10KDa). The NanoMIPs solution was dialysed for eight hours, and the water was changed every two hours. After purification, NanoMIPs were collected and used for sensor preparation. The concentration of NanoMIPs was calculated by evaporating 1 mL and weighing the remaining solid (1.0 mg mL-1).

3.2. Fabrication of the electrochemical sensor

The SPPEs were cleaned prior to functionalization using nitrogen plasma. The electrodes were then incubated in 5% water and 6% APTES in ethanol for 4 h, as shown in Fig. S3, followed by cured at 120 °C for 30 min. The immobilization of NanoMIPs on SPPE was carried out by carbodiimide cross-linker chemistry. For this, SPPE were drop-coated with 50 μL of a mixture of EDC (0.1 mg mL^{-1}), NHS (0.15 mg mL^{-1}) and 50 μL of NanoMIPs (0.5 mg mL^{-1}) as shown in Fig. S3. Prior to electrochemical measurements, the sensor was rinsed with ultrapure



Scheme 1. (a) Synthesis of Metformin imprinted nano-actuators, (b) representation of the sensor response during the voltammetry analysis. The electrochemical response is represented by plotting the potential against the current. The black dotted curve represents the nanoMIP with empty cavity. The red curve on top represents the nanoparticle with a full cavity, after recognition of the analyte.

water to remove any residual mixture.

3.3. Sensor optimization

Optimization of the electrochemical sensor by maximizing the response towards Metformin in terms of sensitivity was performed by studying: (a) the APTES concentration during the functionalization of a sensor (3%, 6% and 9%), (b) immobilization time (2, 4 and 20 h), and (c) NanoMIPs concentration (0.3, 0.5 and 1.0 mg mL^{-1}).

3.4. Electrochemical sensing of Metformin

Electrochemical sensors were investigated using differential pulse voltammetry (DPV) in the potential range from − 0.4–0.4 V (vs Ag/AgCl), the scan rate of 33 mV s^{-1}, modulation amplitude 200 mV, modulation time at 20 ms and step potential of 50 mV. Firstly, the sensor was tested in the blank solution (5 mM PBS), and then spiked Metformin solutions were analyzed in a concentration range of 100–2000 pM in 5 mM PBS buffer (pH = 7.4). Metformin samples (100 μL) were analyzed by drop casting onto the sensor surface and incubating for 3 min. DPV measurements were then assessed in replicates (N = 3). After measuring the voltammetric sensor response, the data was normalized by calculating the current change response using the following equation: $\Delta I = \frac{Is-Ib}{Ib}$, where "ΔI" represents the current change response, "Is" indicates current response of the sample, and "Ib" current response for the blank, background signal (buffer or plasma). Thus, calibration plots were obtained by representing a normalized current change response (ΔI) against the analyte concentration. The limit of the detection (LOD) was calculated conventionally from calibration curves. The limit of the blank (LOB) is the apparent concentration corresponding to the response for a blank sample containing no analyte (native plasma), and calculated by LOB = [mean of blank samples + (1.645 × standard deviation (SD) of blank samples)]. Therefore, LOD is defined as the lowest concentration of analyte in a sample, and it is calculated by LOD = [LOB + (1.645 × SD of samples with low concentration)]. [44–47].

3.5. Determination of Metformin in plasma

Lyophilized human plasma (P9523, Sigma-Aldrich) was reconstituted (5 mL of 5 mM PBS, pH 7.4), then mixed with a vortex for 3 min. Then, centrifuged at 3900 rpm for 5 min and filtered with a syringe filter (25 mm, 0.45 μm, Whatman). Subsequently, plasma was spiked with Metformin in the concentration range of 100–2000 pM.

4. Results and discussion

4.1. Molecular design of the smart nano-actuators

Polymer's recognition cavities specific for Metformin were designed by the screening of a database of common functional monomers used in MIP synthesis using the Leapfrog automated method available in Sybyl. [48,49] Metformin was initially minimized to a 0.01 kcal mol^{-1} A^{-2} gradient using the default Tripos force field and an applied dielectric constant of 80 to simulate an aqueous environment. In addition, the geometry of each monomer was previously minimized and refined by applying Powell's minimization method, with Tripos force fields and charges Gasteiger-Hückel charges, to a minimum energy of 0.001 kcal mol^{-1}. The database used consisted of 60 functional monomers, representing charged and neutral forms of each monomer. The screening was performed for 60,000 iterations, identifying the most suitable interaction between monomers and Metformin and evaluated according to the binding scores. [50] The resulting binding scores revealed that the Metformin interacts preferably with the EGMP (−58.81 kJ mol^{-1}), TFMAA (−38.02 kJ mol^{-1}), and ITA (−36.36 kJ mol^{-1}) functional monomers. The interaction between the functional monomers and the target is drive by hydrogen bonds and electrostatic interactions. The value of these interactions is reported trough binding scores. The minimized 3D structure of Metformin and the interaction of the Metformin-EGMP are shown in Fig. S4. The interaction of Metformin with EGMP, ITA, and TFMAA is shown in Fig. 1. These results were obtained considering the solvation effects and interactions at physiological pH = 7.45.

The monomers' interaction results in the formation of complexes that drive the recognition of the analyte. Thus, after the polymer synthesis and template removal, in the Metformin-recognition sites will be created. Consequently, Metformin will interact with polymer nanoparticles through the recognition cavities. Herein, the role of the TFMAA monomer in the recognition cavity was studied. Two polymer compositions were studied-, one that includes TFMAA is named as NanoMIP-1, and a second formulation that excludes this monomer, known as NanoMIP-2, as shown in Fig. 1.

4.2. Characterization of polymer nanoparticles

The chemical structure of polymers was determined using ATR-FTIR as shown in Fig. S5. NanoMIP-1 presented the N-H ν(s) band at 3268 and 1550 cm^{-1}, C-H (stretching) at 2924–3066 cm^{-1}, and the carbonyl C═O ν(s) can be seen at 1719 cm^{-1}. Furthermore, the bands at 1149 cm^{-1} and 1122 cm^{-1} correspond to C-O and–C-N stretching, respectively. Similarly, NanoMIP-2 spectra present the characteristic bands of the polyacrylamide network. Bands from C-H ν(s) were observed at 2924–2953 cm^{-1}, C═O ν, and N-H δ were observed at 1721 and 1550 cm^{-1}, respectively. The CH2 ν(s) signals appeared at 1379 cm^{-1}.

Moreover, the bands at 1147 cm^{-1} and 1052 cm^{-1} represented C-O ν(s) and –C-N ν(s), respectively. Both nanoparticle formulations presented characteristic signals from polyacrylamide polymer nanoparticles. The only notable difference is the appearance of two additional peaks at 1124 cm^{-1} and 1246 cm^{-1} from NanoMIP-2, which may be attributed to the stretching vibrations C−F bonds.[51] The hydrodynamic diameter of the particles was measured using DLS analysis. The DLS results displayed a diameter of 165 ± 17.5 nm for NanoMIP-1 and 159 ± 1.8 nm for the NanoMIP-2, respectively as shown in Table S2. The polydispersity indexes (PDIs) provide information about the uniformity and mono-dispersive nature of nanoparticles. The PDIs of NanoMIP-1 and NanoMIP-2 were 0.39 and 0.16 respectively, which indicates a homogeneity and uniform distribution of polymer nanoparticles. TEM measurements displayed a diameter of 10.8 ± 1.9 nm for nanoMIP-1 and 14.7 ± 3.8 nm for the NanoMIP-2, respectively as shown in Fig. 2. The analysis revealed that the hydrodynamic diameter measured by DLS is approximately ten times higher due to the aggregation and swelling of particles in the solution.

4.3. Sensor optimization

The nano-actuators were immobilized covalently on SPPE using a cross-linker APTES. The concentration of the cross-linker was optimized. These results revealed that a solution comprising 6% APTES in ethanol gives a better sensor response than those obtained with 3% and 9% APTES, as shown in Fig. S6. During optimization, incubation time 20 h and NanoMIPs concentration 0.5 mg mL^{-1} were selected. This finding seems to be a trade-off between the number of immobilized NanoMIPs and the concentration of APTES, resulting in a higher sensor response. Conversely, excess in the APTES concentration results in the deposition of an insulating layer hindering the electron transfer and decreasing sensor response. Additionally, the sensor performance was also evaluated by varying the NanoMIPs concentrations from 0.3 to 1.0 mg mL^{-1}. For the optimization, incubation time (20 h) and APTES concentration (6%) were kept constant. The results obtained, revealed that the sensor sensitivity was enhanced two-fold using a nanoparticles concentration at 0.5 mg mL^{-1}; a lower concentration of NanoMIPs (0.3 mg mL^{-1})

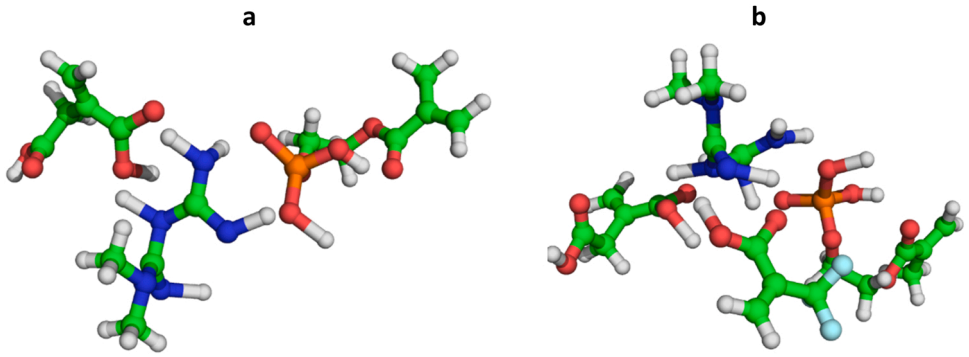


Fig. 1. Molecular modelling of the monomer interaction with Metformin for: (a) NanoMIP-1 involving ITA-Metformin-EGMP complex, and (b) NanoMIP-2 comprising ITA-Metformin-EGMP-ITA-TFMAA complex.

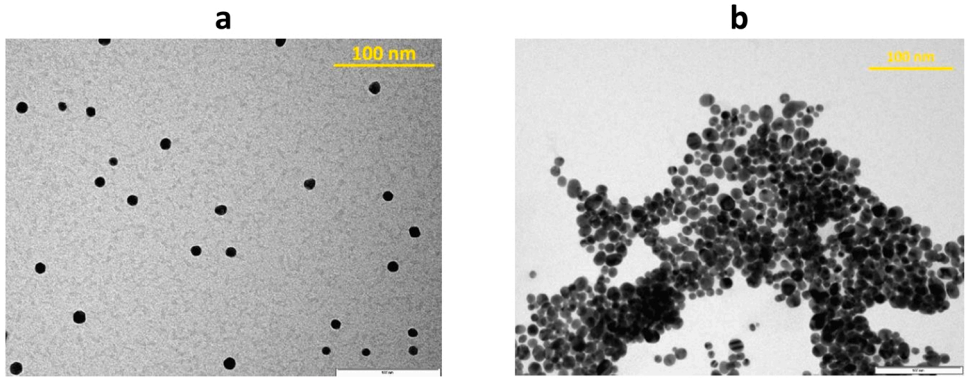


Fig. 2. TEM images of metformin-NanoMIPs: (A) NanoMIP-1 and (B) NanoMIP-2, both scale bars at 100 nm and 50 K magnification.

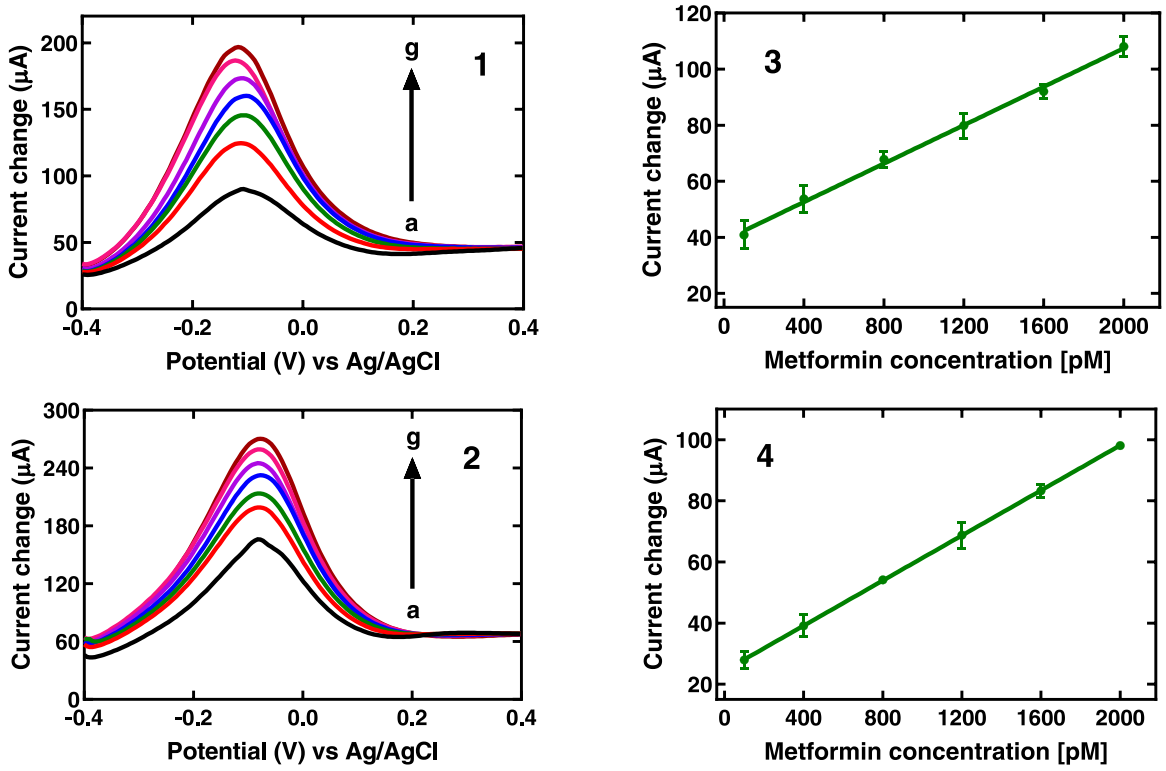


Fig. 3. DPV sensor response from (1) NanoMIP-1, (2) NanoMIP-2 to metformin concentration to (a) 100, (c) 400, (d) 800, (e) 1200, (f) 1600, and (g) 2000 pM in 5 mM PBS. Calibration plots from DPV response from (3) NanoMIP-1 and (4) NanoMIP-2, measurements RSD at 2.5% and N = 3.

reduced the sensor response due to the insufficient number of nanoparticles while a higher concentration of nanoparticles (1.0 mg mL^{-1}) lead to aggregation which decreased the sensor response, as shown in Fig. S7. Finally, the time required for nanoparticle immobilization on the sensor was also studied. For the optimization, the incubation time was varied (2, 4 and 20 h) and NanoMIPs (0.5 mg mL^{-1}) and APTES (6%) concentration were kept constant. Both NanoMIPs demonstrated an optimal immobilization time of 4 h, as shown in Fig. S8. The optimized parameters and the sensor performance are shown in Table S3.

4.4. Nano-actuator sensor response

The voltammetric sensor response for both compositions presented a linear response, directly proportional to the Metformin concentration as shown in Fig. 3. The redox marker (ferrocene) in the polymer network confers electroactive properties to NanoMIPs. The sensor exhibited a current increase when exposed to the Metformin concentrations. Possibly, the actuation mechanism is triggered by the analyte, causing swelling and a change in polymer conformation. These changes increase the surface area and, volume, which and prompt the electron transfer between ferrocene and electrode surface. This nanoparticle actuation increases the electron transfer, which is translated into an increase in the sensor response. The sensitivity displayed was 34.2 ± 1.3 nA pM^{-1} (R^2 =0.987) and 36.9 ± 0.9 nA pM^{-1} (R^2 = 0.998) for NanoMIP- 1 and NanoMIP-2, respectively. The LOD for NanoMIP-1was found at 13.7 pM and for nanoMIP-2 at 9.0 pM. Therefore, NanoMIP-2 presents better performance as shown in Fig. 3.

4.5. Selectivity, performance and validation of the method

Metformin is used frequently in a binary combination with other drugs such as Sitagliptin for the treatment of DMT2. Therefore, the cross-reactivity of the sensor was tested by measuring the sensor response against common drugs (Paracetamol and Sitagliptin), and then compared to the Metformin response as shown in Fig. 4. NanoMIP-1 sensor response displayed a response to Sitagliptin and Paracetamol at 37% (R^2 = 0.72) and 9% (R^2 = 0.41), respectively. In contrast, the NanoMIP-2 sensor exhibited a response to Sitagliptin and Paracetamol at 5% (R^2 = 0.31) and 7% (R^2 = 0.27), respectively as shown in Table S4.

These results indicate that the TFMAA monomer present in NanoMIP-1 increases the cross-reactivity to Sitagliptin. This limited selectivity is due to the interactions between fluorine groups of the polymer and the Sitagliptin (dipole and polar interactions), which do not occur with NanoMIP-2. Moreover, the NanoMIP-2 sensor is 14 fold more responsive to Metformin when compared to Sitagliptin response, as summarized in Table S4-S5. Therefore, from these results, it can conclude that the NanoMIP-2 sensor displayed better selectivity when compared to the NanoMIP-1 sensor. Thus, NanoMIP-2 sensors could be used effectively for monitoring Metformin in complex matrices and in the presence of other drugs. Accordingly, the NanoMIP-2 sensor was used for further studies.

Additionally, the affinity of nanoMIP-2 was assessed by SPR measurements as shown in Fig. S9. The metformin-imprinted nanoMIP shown 3.5 × 10^6 times higher response to metformin when compared to paracetamol. Since NIP is not possible to obtain trough solid phase synthesis. The imprinting effect was tested by preparing a MIP particle using the same polymer composition but a different template. Therefore, the same polymer formulation was used to synthesize particles imprinted with paracetamol. Afterwards, the SPR interaction between metformin and the paracetamol-imprinted particle was measured. As a result, the SPR showed no interaction between metformin and the paracetamol-imprinted particles. (Fig. S9). This demonstrate the imprinting effect on the nanoMIPs.

Subsequently, nanoMIP-2 was tested on biological samples. The sensitivity displayed for NanoMIP-2 in plasmas was found at 31.5 ± 0.7 nA pM^{-1} (R^2 =0.998) and RSD at 1.7%. The LOD was found at 13 pM and 9 pM for spiked buffer and plasma, respectively. The plasma effect on the sensor was found to drop the signal by 13.2%, but it did not significantly affect the sensitivity and LOD as shown in Fig. 5. Moreover, the characteristic redox behavior of the NanoMIPs in the plasma is kept and does not affect the DPV signal. Therefore, the present sensor is highly sensitive and selective, allowing monitoring of Metformin at detection limits overcoming the classical challenges of the point of care diagnostic, such as efficiency, simplicity, time of measurement (3 min), availability of the user- friendly platform, and cost-effectiveness.

Additionally, the sensor response was tested against endogenous interferences present in human plasma at biological relevant concentrations. For that purpose, human plasma was spiked with 1200 pM Metformin and measured in presence of 68 μM Ascorbic acid, 315 μM Uric acid, 22.3 nM Folic acid and 196 pM Dopamine, respectively. The sensor response was not influenced by neither ascorbic acid (9.3%), Uric acid (7.7%), Folic acid (5.2%) nor dopamine (2.7%) as shown in Fig. S10. Thus, no cross reactivity was found by these endogenous biomolecules and do not affected significantly to the sensor response.

Accuracy, repeatability, precision and bias% were calculated according to the ICH guidelines [52,53] and assessed by using 24 determinations over six different concentrations levels with four replicates each, covering in the range 100–2000pM in human plasma. Accuracy was calculated by measuring the similarity between the measurement and the actual value, It was determined at RSD 3.1%. Precision was calculated by determining the degree of recurrence of the values obtained by repeating the same measurements under the same conditions, which was found at RSD 4.5% in a 95% confidence interval. Repeatability was measured over seven sensors by measuring three different concentration levels (500, 1000, and 2000 pM) with four replicates. The repeatability was found at RSD 3.1–6.3%. The Bias from these

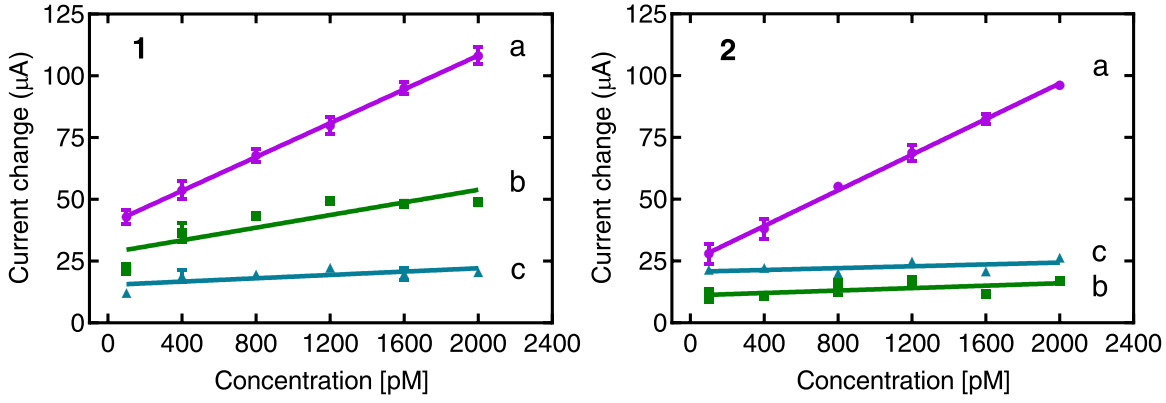


Fig. 4. Selectivity study for (1) NanoMIP-1 and (2) NanoMIP-2 sensor. Calibration plots correspond to DPV sensor response to (a) Metformin, (b) Sitagliptin, and (c) Paracetamol in a concentration from 100–2000 pM in 5 mM PBS, measurements at RSD at 3.7% and N = 3.

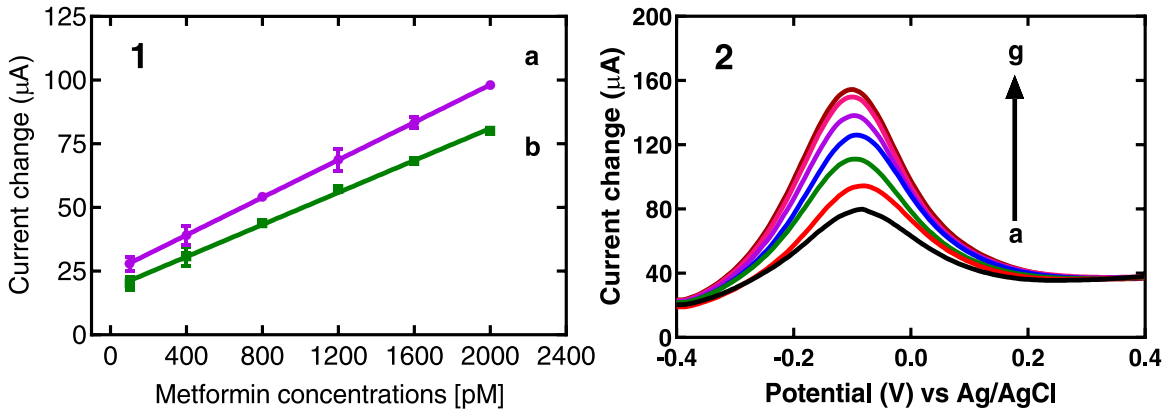


Fig. 5. (1) Calibration plots for NanoMIP-2 sensor in (a) 5 mM PBS and (b) plasma spiked with Metformin. (2) DPV response of the NanoMIP-2 sensor in plasma spiked with Metformin in a concentration from 100–2000 pM, measurements at RSD at 1.7% and N = 3.

experiments was found at 2.8%. Recoveries were estimated at 100.3–110.6% and determined by comparing the sensor's response to standard solution and spiked plasma (Table S6).

4.6. Robustness, storage, and stability of the sensor

The sensor robustness was tested at different temperatures and buffer concentrations in a pH range. As shown in Fig. S11, the sensor displayed adequate performance at 22–34 °C (RSD 3.5–8.5%). The sensor compatibility with different buffers was tested by measuring the response to a 1000 pM Metformin in various buffers and pH ranges, as shown in Fig. S12. These buffers display a satisfactory response, being the most suitable the PBS (RSD 3.1%) with a sensor response at 92–100% when compare to HEPES (RSD 4.3%) at 91–99% and Bis-Tris Propane (RSD 5.2%) at 83.5–99.4%. The study also showed that pH range 7–8 is the best for Metformin sensing. Besides, different concentrations of PBS (1–11 mM) were also tested to measure the electrolyte concentration's impact on the sensor response (Fig. S13). The description of the buffer composition is shown in Table S7. The concentration selected due to the performance is 5 mM PBS (RSD 4.1%). To evaluate the storage stability of NanoMIPs-2, seven sensors were fabricated under identical conditions and then stored at room temperature (20–22 °C) and 50% relative humidity. Each sensor (1−7) was tested independently, every 30 days for a period ranging up to 180 days. DPV response was used to prepare calibration plots in a concentration range from 200 to 2000 pM of Metformin in 5 mM PBS. The stability of the sensors is summarized in Fig. S14, and Table S8**.** Herein, the average response and standard deviation are displayed per sensor per day. After 30 days the drop in sensitivity was 7.5% and after 120 days (17.5%) the sensitivity stabilized with no significant changes in the sensor performance. After that period, the sensors remained operating efficiently, RSD at 6.8%. This analysis demonstrates that the sensor performance follows the industrial accuracy standards (ISO-151917), which states that 95% of results should be within ± 15% of a laboratory standard.[54–56].

4.7. Comparison of available metformin electrochemical sensors

The sensor described shows satisfactory sensitivity, selectivity, and LOD for Metformin detection as compared to other sensors, as shown in Table 1. The present sensor technology is cost-effective, with a simple fabrication, low detection limit (9 pM), rapid response (3 min), selective, satisfactory stability, and useful in the complex bio-fluids analysis as compared to other sensors as shown in Table 1. These characteristics are essential within clinical laboratories for Metformin monitoring. [57] Compared to conventional HPLC methods do not meet these requirements. [58] The presented here sensor technology offers a rapid response, higher sensitivity and much lower detection limit, and a broader linear dynamic range for Metformin monitoring in complex

Table 1
Summary of the available Metformin sensors tested in biological samples.

Working Electrode	Detection method	Linear range (μM)	LOD (nM)	RSD (%)	Sample	Ref
SBA-15-Cu/CPE	DPV	0.1–65	30	>3.6	Serum, Urine	[60]
Cu/CILE	SWV	1–4000	500	2.4	Urine	[61]
Cu-AC/CPE	DPSV	0.05–60	9	>4	Urine	[62]
n-NC/CPE	Amperometry	4–63	450	>4	Serum, Urine	[63]
Fe-Cu/TiO_2/CPE	SWAdSV	0.015–75	3	3.5	Urine	[64]
Zn-Fe_2O_4-CuO /AuNPs/GCE	DPV	0.001–1	0.3	2.7	Serum	[65]
γ-Fe_2O_3 @HAP/Cu /CPE	ASDPV	1–80	14	>4	Urine	[66]
PGE	SWV	2.76–24.8	9.03	>4	Urine	[67]
HMDE	SWV	0.1 − 2	18	8.4	Urine	[68]
GNF-PMB/SiO_2-F	CV	100–1000	0.1	1.5	Urine	[69]
PY/CPE	DPV	1.6 − 5.6	91.4	1.8	Urine	[70]
NanoMIPs/SPPE	DPV	0.0001–0.002	0.009	1.7	Plasma	This Work

Mesoporous silica material functionalized by copper ion (SBA-15-Cu), carbon paste electrode **(**CPE), copper hydroxide nanoparticle-modified carbon ionic liquid electrode (Cu/CILE), copper loaded activated charcoal (Cu-AC), nickel oxide nanotubes-carbon micro-particles/nafion nanocomposite (n-NC), iron, copper, titanium oxide nanoparticles (Fe-Cu/TiO_2), zinc ferrite and copper oxide nanostructure (Zn-Fe_2O_4-CuO), gold nanoparticles (AuNPs), glassy carbon electrode (GCE), hematite-hydroxyapatite-copper nanocomposite (γ-Fe_2O_3 @HAP/Cu), pencil graphite electrode (PGE), hanging mercury drop electrode (HMDE), graphene nano-flakes-polymethylene blue/ fluorine-doped tin oxide glass electrode (GNF-PMB/SiO_2-F), pyrogallol (PY), square wave voltammetry (SWV), differential pulse stripping voltammetry (DPSV), square wave adsorptive stripping voltammetry (SWAdSV), adsorptive stripping differential pulse voltammetry (ASDPV), cyclic voltammetry (CV).

biological mixtures. For instance, HPLC methods exhibit LOD values ranging from 20 ng mL^{-1} (120.7 nM) to 62 ng mL^{-1} (374.3 nM). [24, 59] Moreover, HPLC working ranges are usually between 0.2 and 2.5 μg mL^{-1} (1.20–15.09 μM) and 0.125–2.5 μg mL^{-1} (0.754–15.09 μM). [24,59] Furthermore, with our sensor technology, sample preparation is easier because it takes fewer steps, and analysis is completed in a shorter time. Most of the previously mentioned classic HPLC methods do not meet these aspects. [58].

In contrast with classical electrochemical detection of Metformin, this method relies on the actuation of the NanoMIPs. The main disadvantage in the classic electro-oxidation of Metformin is the poor selectivity, the cross-reactivity in biological samples, parasitic reactions and sensor poisoning. In this regard, electroactive NanoMIPs are computationally designed to recognize the target, assuring their sensitivity and selectivity. They are in fact both, recognition elements and transducers. These smart nano-actuators,represents the most generic, versatile, scalable, and cost-effective approach to the manufacture of synthetic molecular receptors. In addition, these polymer nanoparticles are much more robust than their biological counterparts (antibodies, enzymes etc.) in their stability to harsh conditions (temperature, pH, pressure, and organic solvents).

5. Conclusion

Computationally designed nanoparticles were synthesized for Metformin using molecular imprinting. The sensor was prepared by integrating nanoparticles on screen-printed electrodes. Conditions affecting the immobilization of particles, such as cross-linkers, incubation time and particle concentration, were optimized to enhance the sensor's sensitivity. Moreover, selecting suitable monomer composition allowed us to tune the sensor's selectivity. Metformin sensing in the presence of other drugs in human plasma samples was demonstrated. In addition, the sensors showed high stability (120 days) and accuracy (RSD 6.8%). The practical aspects of the technology as sensitivity, rapid response, and portability, make it suitable for diagnostics at point-of-care testing. Future work will focus on improving the fabrication by employing printing techniques and studying nanoparticle integration utilizing graphene ink.

CRediT authorship contribution statement

Isma Haq: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, **Alvaro Garcia Cruz**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, **Sabrina Di Masi**: Formal analysis, Writing – original draft, Writing – review & editing, **Todd Cowen**: Formal analysis, Investigation, Data curation, **Natalie S. Allcock**: Investigation, **Cosimino Malitesta**: Writing – review & editing, **Adnan Mujahid**: Resources, Writing – review & editing, **Tajamal Hussain**: Resources, Writing – review & editing, **Elena Piletska**: Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition, **Sergey A. Piletsky**: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to thank the funding from the European project BorderSens (Grant agreement ID: 833787).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.132928.

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