**Design and fabrication of a smart sensor using *in silico* epitope mapping and electro-responsive imprinted polymer nanoparticles for determination of insulin levels in human plasma**

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**Abstract:** A robust and highly specific sensor based on electroactive molecularly imprinted polymer nanoparticles (nanoMIP) was developed. The nanoMIP tagged with a redox probe, combines both recognition and reporting capabilities. The developed nanoMIP replaces enzyme-mediator pairs used in traditional biosensors thus, offering enhanced molecular recognition for insulin, improving performance in complex biological samples, and yielding high stability. Also, most of existing sensors show poor performance after storage. To improve costs of the logistics and avoid the need of cold storage in the chain supply, we developed an alternative to biorecognition system developing system that relies on nanoMIP. NanoMIP were computationally designed using “in-silico” insulin epitope mapping and synthesized by solid phase polymerization. The characterisation of the polymer nanoparticles was performed by transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier-Transformed Infrared (FT-IR) and surface plasmon resonance (SPR). The electrochemical sensor was developed by chemical immobilisation of the nanoMIP on screen printed platinum electrodes. The insulin sensor displayed satisfactory performances and reproducible results (RSD=4.2%; *n*=30) using differential pulse voltammetric (DPV) in the clinically relevant concentration range from 50 to 2000 pM. The developed nanoMIP offers the advantage of large number of specific recognition sites with tailored geometry, as the resultant, the sensor showed high sensitivity and selectivity to insulin with a limit of detection (LOD) of 26 and 81 fM in buffer and human plasma, respectively, confirming the practical application for point of care monitoring. Moreover, the nanoMIP showed adequate storage stability of 168 days, demonstrating the robustness of sensor for several rounds of insulin analysis.

**Keywords:** molecularly imprinted polymers, artificial receptors, insulin, nanoparticles, voltammetric sensor

1. **Introduction**

Insulin is an anabolic hormone produced by pancreatic beta cells that regulates carbohydrate homeostasis, lipid metabolism, and influences protein synthesis.(Santen et al. 1972; Wilcox 2005) Insulin promotes the conversion of small molecules in the blood into large molecules inside the cells. Moreover, it has a vast array of biological functions, but the most important is in the metabolism of glucose. Insulin is synthesized as pre-proinsulin and processed to human proinsulin C-peptide (HPC). HPC is then converted to insulin and C-peptide and then stored for release.(Fu et al. 2013) The Insulin, C-peptide and insulin-like growth factor 1 (IGF1) levels are correlated.(Verhaeghe et al. 1993)

The insulin testing levels are important to identify patients with dysfunctions such as hypoglycaemia, insulinoma, autoimmunity, etc.(McPherson and Pincus 2017) Therefore, insulin plays a major role in glucose metabolism and its determination is of great value in the diagnosis and control of diabetes.(Sabu et al. 2019) Insulin resistance may be measured by directly observing at insulin mediated glucose uptake in the basal or post-stimulated state.(Wilcox 2005; Williamson and Snyder 2014) There are a variety of approaches to the laboratory assessment of insulin resistance.

The most common used analytical techniques are the insulin immunoassays, but besides the cost of development these have a number of drawbacks. Firstly, a limited specificity arises due to cross-reactivity with proinsulin, reducing the reliability of insulin measurement in clinical settings. Additional cross-reactivity with HPC, IGF1 and IGF2, though minimal, varies with the specific brand of the testing toolkit and technique used. (Blum et al. 1988; Dayaldasani et al. 2015; Sapin 2002) Further to this cross-reactivity with interferent structures is the lack of discrimination between endogenous and exogenous insulin in the standard test, and a lack of standardization generally in assay procedures, preventing comparison between studies and allowing only qualitative analysis.(Staten et al. 2010) Unfortunately, these methods require the implementation of laborious protocols with expensive and specialized instrumentations.

Alternatively, electrochemical sensors offer several advantages compared to other analytical methods.(Sabu et al. 2019) There is a lot of interest especially for detection of specific biomolecules over the recent years due to their high sensitivity, selectivity, wide linear range, low detection limit, simple preparation and quick response.(Hovancová et al. 2017) In addition, the electrochemical sensors enable to production of a simple and portable device for the real time monitoring of biological markers in human plasma and urine samples.(Bollella et al. 2017)

Recently, sensors for the determination of insulin using modified surfaces electrodes with nanomaterials and specific biomimetic receptors have been reported.(Cheng-Jun et al. 2017; Šišoláková et al. 2020) The most commonly used biomimetic receptors for sensors are the molecularly imprinted polymers (MIP)(Ahmad et al. 2019; Selvolini and Marrazza 2017), which they have been widely reported as specific and selective receptors in the development of insulin sensors.(Cheng-Jun et al. 2017; Prasad et al. 2010) However, existing methods present disadvantages due to the matrix effect and cross-reactivity. Matrix effect is the signal distortion in a sensor reading, that occurs during the analysis of biological samples (urine, plasma, saliva etc.) caused by the presence of cells, biomolecules, heterogeneity in ionic strength, pH, temperature etc.(Gaster et al. 2009) To summarize, these strategies are not suitable for real scenarios and near patient testing due to the complexity of the systems and their difficult industrial production. Also, these approaches are not suitable due to the lack of selectivity and cross-reactivity with the biological samples.

In the sensor presented here, the solid phase approach was employed for the preparation of synthetic recognition elements known as electroactive molecularly imprinted polymer nanoparticles (nanoMIP). The nanoMIP possess well-defined recognition sites towards the insulin target followed. The use of electrochemically active ferrocene monomer acting as a redox probe eliminates the need of mediators or enzymes employed in traditional biosensors. Thus, the nanoMIP combines both recognition and reporting capabilities leading to a higher sensitivity and low cross reactivity in biological samples. Conversely, traditional methods involve the monitoring of the redox activity of an analyte, which is affected by interference and the nature of the sample. The present work allows these issues to be overcome.

Also, an optimized protocol for covalent immobilization of nanoMIP on screen-printed platinum electrodes (SPPE) is described as shown in **Scheme 1**. To summarise, a highly selective, sensitive and stable insulin sensor is reported, which is fully compatible with mass industrial production and can be used in real scenarios for near patient test.

1. **Materials and Methods**
	1. **Chemicals**

Allylamine, ammonium persulfate (APS), 1,2-bis(triethoxysilyl)ethane (BTSE), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ferrocenylmethyl methacrylate (FcMMA), glutaraldehyde, N-hydroxysuccinimide (NHS), N,N’-methylene-bis-acrylamide (MBA), 12-mercaptododecanoic acid (MDA), 2,2,2-trifluoroethyl methacrylate (TFEMA), (N,N-diethylaminoethylmethacrylate) DEAEM, N-isopropylacrylamide (NIPAM), 2-hydroxyethyl methacrylate (HEMA), 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNSCl), N-tert-butylacrylamide (TBAM), itaconic acid (ITA), N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane (DAMO), phosphate buffered saline (PBS), sodium hydroxide (NaOH) and tetramethylethylenediamine (TEMED), were purchased from Sigma-Aldrich, UK. N-(3-Aminopropyl) methacrylamide hydrochloride (NAPMA) was purchased from PolySciences Inc.; Glass beads A-class 2429 (SPHERIGLASS®) with 150 – 200 µm diameter), were from Potters Industries LLC. Ethylene glycol methacrylate phosphate (EGMP) was acquired from Alfa-Chemistry USA. Double-distilled ultrapure water (Millipore, UK) was used for all experiments. All chemicals and solvents were analytical or HPLC grade and used without any purification. Pierce Ninhydrin and bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (UK).

* 1. **Instrumentation**

A nitrogen plasma was used to activate and clean the surface of screen-printed electrodes and the gold chips for SPR measurements (Emitech, K1050X RF Plasma Cleaner, 50 W, 13.56 MHz RF for 5 min). Transmission electron microscopy (TEM) images were obtained by using JEOL JEM-1400 (accelerating voltage of 100kV) coupled with a Megaview III digital camera with iTEM software. Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano (Nano-S) from Malvern (UK). For these measurements, 1mL solution of nanoparticles was ultra-sonicated for 10 min in order to disrupt potential agglomerates. FT-IR analysis was performed using an Alpha platinum- ATR FTIR spectrometer (Bruker, UK).

The screen printed platinum electrodes (SPPE) type DRP-550 with dimensions of 3.4 cm x 1.0 cm x 0.05 cm (length x width x height) were purchased from Metrohm (UK) and used for the sensor development. The SPPE comprised a platinum working and counter electrode, and a Ag/AgCl refence electrode. All the electrochemical measurements were performed by using PalmSens4 Potentiostat / Galvanostat / Impedance Analyzer equipped with a cable connector for screen-printed electrodes purchased from Alvatek (UK). The data acquisition was controlled by the PSTrace 5.0 software (Alvatek, UK).

* 1. **Glass beads activation and silanization**

200 g of glass beads (150-200 μm) were activated by boiling in 250 mL of 4 M NaOH for 15 min, then washed thoroughly with double-distilled water. After that, glass beads were washed two times (250 mL) with 5 mM PBS buffer, resulting in neutral pH. Glass beads were then washed twice with water and acetone (100 mL), subsequently dried under vacuum for 5 min and cured for 15 min at 70 °C and 60 min at 120 °C. Silanization of glass beads was achieved using a reflux system in a solution of 6% (v/v) of DAMO and 0.24% (v/v) of BTSE in anhydrous toluene (1.3 mL of solution per g of glass beads) for 8 h. Successively, the system was cooled down and the glass beads were washed with 200 mL of acetone, dried under vacuum for 5 min and cured at 120 °C for 60 min. Silanization was verified by fluorescence. For this verification, 0.5 g of glass beads were incubated in a 1 mL solution of DNSCl (40 mM in ethanol) for 2 h in dark condition. The solution was then poured and rinsed with ethanol. Finally, the presence of silanes on the glass beads was verified by fluorescence assessment under UV sources (365 nm).

* 1. **Preparation of glassbeads with immobilized insulin**

Glass beads (40 g) were incubated in 50 mL of 7% (v/v) of glutaraldehyde in PBS (10 mM, pH=7.2) for 2 h. Glass beads were then rinsed with double-distilled water and incubated overnight at room temperature in an insulin solution (0.5 mg mL-1 dissolved in 5 mM PBS solution, pH = 7.2). Successively, glass beads were rinsed with double-distilled water and incubated in 50 mL of 0.1 mM ethanolamine in 5 mM PBS for 15 min. Subsequently, glass beads were washed with distilled water and then incubated in a 1 mg mL-1 sodium cyanoborohydride solution dissolved in 0.01 M PBS (1.5 mL of solution per g of beads) for 30 min at room temperature. Finally, the glassbeads with immobilized insulin were filtered and rinsed with ultrapure water, dried and stored at 4 °C until use.

* 1. **Synthesis of Insulin molecularly imprinted nanoparticles (nanoMIP)**

The preparation of nanoMIP was carried out by the free radical polymerization using solid phase synthesis approach as it was described earlier.(Garcia-Cruz et al. 2020) The monomers used in nanoMIP synthesis constituted a “standard” composition, which included functional monomers that are capable to form specific molecular interactions with a variety of the peptide side chains of the protein and as it was shown earlier, could be used for imprinting of protein sequences.(Piletska et al. 2019) These monomers include the cross-linkers NIPAM, MBA, TBAM, and the functional monomer NAPMA. Additional insulin-specific monomers were also considered, particularly allylamine, HEMA, EGMP, ITA and TFEMA, which were selected using molecular modelling.

To optimize the polymer properties, different formulations were selected, as listed in **Table S1**. As result, three different nanoMIP were synthetized. All components were dissolved and sonicated individually in 1 mL of DMSO, then added to 50 mL double-distilled ultrapure water. For example, nanoMIP 3 formulation comprised: NIPAM (20 mg, 0.17 mmol), NAPMA (6 mg, 0.03 mmol), MBA (52 mg, 0.34 mmol), allylamine (9 mg, 0.15 mmol), EGMP (16 mg, 0.08 mmol), FcMMA (8 mg, 0.02 mmol), TFEMA (29 mg, 0.17 mmol) and HEMA (4.5 mg, 0.03 mmol). Afterwards, 50 mL of this polymerisation solution was degassed with nitrogen and sonicated for 5 min and added to 60 g of glass beads modified with the insulin. The polymerisation reaction was initiated by addition of 0.5 mL (60 mg mL-1) of ammonium persulfate and 30 μL mL-1 of TEMED. The polymerisation was carried out at room temperature for 2 h. Subsequently, the polymerisation was stopped by adding 50 mg of sodium nitrite and by purging with oxygen for 5 min.

Afterwards, the glass beads were transferred into a 60 mL solid phase extraction cartridge fitted with polyethylene frit (20 µm porosity). Unreacted monomers and other low-affinity materials were removed by eluting with cold water at 0 °C (5 x 50 mL). Subsequently, the cartridge was filled with 20 mL of ethanol and warmed in a water bath at 60 °C for 10 min and this solution was collected. Finally, the remaining fractions of high-affinity nanoMIP were collected by eluting with water at 60 °C (4 x 10 mL) as shown in **Scheme 1**.

**Scheme 1.** Synthesis and elution of molecularly imprinted nanoparticles by thermo-elution process and their integration on screen printed platinum electrodes.

The purification of nanoMIP by thermal elution is possible due to the presence of the poly-NIPAM, which confers thermoplastic and thermosensitive properties. Thus, residues are removed from the solid phase by elution at low temperature. In that way, at high temperature, the non-covalent interactions between the template insulin and the formed nanoMIP are disrupted, making possible the extracted of nanoMIP from the solid phase.

The imprinted nanoparticles were purified using ultrafiltration. The ninhydrin test confirmed that there are no trace of insulin in the collected nanoparticles. Also, the BCA protein assay was used accordingly to the manufacturer’s protocol to evaluate the presence of the insulin in dialysed samples of nanoMIP. Typically, if traces of insulin were present, further dialysis was applied followed by BCA test. Once the traces of insulin were efficiently removed, the nanoMIP were collected and lyophilized. After that, the resulting solid was weighted and reconstitute in distilled water for further use.

* 1. **Fabrication of the sensor and electrochemical measurements**

Sensors were prepared by immobilisation of nanoMIP on SPPE. For that, SPPE were activated in nitrogen plasma and then silanized by incubating for 1 h in a solution of 6% APTES and 5% water in ethanol and then cured for 30 min at 120 °C. Subsequently, a solution comprising 100 µL of nanoMIP (0.5 mg mL-1) and 100 µL of 7% glutaraldehyde in 10 mM PBS was drop casted on the working surface of the electrode and incubated for 60 min. As a final point, the SPPE modified with insulin specific nanoMIP was rinsed with distilled water and stored at 4 ⁰C until further use.

The electrochemical performance of the sensors was evaluated by using differential pulse voltammetry (DPV) in the potential range from -0.2 to 0.6 V (vs Ag/AgCl), scan rate of 25 mV s-1, modulation amplitude 200 mV, modulation time at 20 ms and step potential of 50 mV. The standard insulin solutions were studied in the clinically relevant concentration range from 50 to 2000 pM in 5 mM PBS (pH=7.2). After measuring the voltammetric sensor response, the data was normalized by calculating the *current change response* using the following equation for calibration plots: $∆I=\frac{ys-yb}{yb}$. Herein, “∆I” represents the *current change response*, “ys” signifies current response of the sample, and “yb” current response for zero concentration (buffer). Limit of detection (LOD) was calculated conventionally from calibration curves (3×SD/slope).

The spiked human plasma sample was prepared as following: Firstly, 0.5 mL of human plasma was diluted in 5 mL of 10 mM of PBS (pH = 7.2). After agitating for 3 min with a vortex, the diluted sample was centrifuged for 3 min at 3500 rpm, and thus the supernatant was recovered and filtered using a syringe containing a microfiltration membrane (with a pore diameter of 0.45 µm PTFE, 25 mm diameter, at 87 psi) Corning Inc. (Germany). Finally, the human plasma was spiked with 50 pM to 2000 pM concentration range of insulin and tested using the developed electrochemical sensor.

1. **Results and Discussion**
	1. **Molecular modelling and epitope mapping**

NanoMIPs compositions were formulated by modifying a standard composition and according to the results of the screening. (Garcia-Cruz et al. 2020) The detailed protocol and conditions used for the epitope mapping and molecular modelling are described in supporting information in section 2. The insulin modelling results displayed six epitope types, summarized in 29 binding interactions categorised as follows: 4 acidic (1 Asp D, 3 Glu), 2 basic (1 His, 1 Lys), 7 aromatic (4 Tyr, 3 Phe), 5 amidic (3 Asn, 2 Gln), 3 hydroxylic (2 Thr, 1 Ser), and 9 aliphatic (2 Leu, 1 Ile, 4 Val, 1 Pro, 1 Ala) as shown in **Table S2**. The strongest binding monomer interactions were obtained for allylamine, MBA and EGMP monomers as shown in **Table S2** and **Figure S1**. A high binding interaction was recorded for allylamine molecular complex for epitope 1 at -488.8 kJ mol-1. MBA (epitope 4) displayed a binding score of -307 kJ mol-1 and EGMP (epitope 2) at -526.6 kJ mol-1. The key monomers formed a cavity for insulin recognition as shown in **Figure S1**. From these calculations, it was concluded that the optimal interaction between insulin, allylamine, EGMP and MBA happen at 1:2:1:1 molar ratio. Based on the modelling results, different nanoMIP compositions were proposed as shown in **Table S1**.

* 1. **Characterization of nanoMIP**

The chemical composition of the nanoparticles (nanoMIP 1, 2, and 3) was evaluated using FT-IR analysis (**Figure S2**). These particles displayed the main characteristic bands of polyacrylamide structure. The bands observed were at 3270 cm-1 for (ν, N-H and O-H), 2943, 2872 and 848 cm-1 for (ν, C-H). The amide bands appeared at 1654 and 1576 cm-1 for the (ν, C=O), 1401 and 1313 cm-1 for (ν, C-N). Besides, (ν, C-O) and (ω, N-H) were observed at 1201 and 1104 cm-1, respectively. The (δ, O-H) was found at 1026 and 987 cm-1.

Nanoparticle size was measured using dynamic light scattering (DLS) with satisfactory low polydispersity index (PDI) indicating uniform distributions of nanoparticles (**Table S3**). For instance, nanoMIP 2 showed an average diameter of 261.2 ± 1 nm (n=6) with a PDI of 0.201. Additionally, nanoMIP were exposed to insulin (500 pM, ~5 kDa) and then washed and dialysed (cut-off membrane ~10 kDa). The resulting particle diameters for nanoMIP 1, 2 and 3 were increased by 14.2%, 22.4% and 17.5%, respectively, when exposed to insulin but not to glucose, albumin or other molecules. Presumably, this specific polymer actuation is due to conformational changes triggered by the insulin, which might also lead to agglomerates. The DLS summary is provided for the different nanoMIP formulations in **Table S3**.

 The morphological characterisation of nanoMIP was performed using TEM. Samples were diluted in ethanol and then dried. The TEM images displayed aggregate spherical uniform nanoparticles (**Figure S3**). TEM measurements for nanoMIP 1, nanoMIP 2, and nanoMIP 3 revealed an average particle size of (210 ± 7) nm, (215 ± 6) nm and (255 ± 5) nm, respectively. The hydrodynamic diameters observed in DLS were superior possibly due to the presence of water molecules and aggregates formed in solution. Also, sample preparation causes aggregation and alteration of the nanostructures.

* 1. **NanoMIP affinity and cross-reactivity**

The affinity and selectivity of the nanoparticles towards the insulin were evaluated in terms of the dissociation constants (Kd) using surface plasmon resonance (SPR). The experimental conditions and method employed for the SPR analysis is described in supporting information section 3. The SPR responses of nanoMIP 1, nanoMIP 2 and nanoMIP 3 were measured in the concentration range from 0.044 to 440 nM of insulin, as shown in **Figure S4**. The obtained Kd for nanoMIP 1, nanoMIP 2, and nanoMIP 3, were calculated at 1 nM (Chi2 = 9), 0.74 nM (Chi2 = 3.3) and 0.9 nM (Chi2 = 1.56), respectively. Those results demonstrated high affinity of nanoparticles towards insulin. The nanoMIP 2 and 3 were selected and then tested against interferents, e.g. human proinsulin C-peptide (HPC) and insulin-like growth factor 1 (IGF1) to evaluate the cross-reactivity as shown in **Figure S5-S6** and summarized in **Table 1**.

The percentage of cross-reactivity was calculated from the Kd values. The nanoMIP 2 interference towards IGF1 and HPC was found at 25% and 0.7%, respectively. Similarly, nanoMIP 3 interaction towards IGF1 and HPC was found at 7% and 0.07%, correspondingly. The interference response is due to some degree of sequence homology between the HPC, IGF1 and insulin. Particularly, the IGF1 presents a sequence homology to insulin (49%), located in the A (62%) and B (40%) chain from insulin. In conclusion, nanoMIP 3 presented the highest selectivity and affinity to insulin, for that reason, it was selected for further experiments (**Table 1**).

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| **Table 1**. SPR response of nanoMIP to different analytes |
| Molecule | **Insulin** | **HPC** | **IGF1** |
| MW (kDa) | 5.8 | 3.6 | 7.6 |
| Number of AA | 51 | 35 | 70 |
| Sequence homology | 100% | 11% | 49% |
| Isoelectric pH | 5.4 | 3.1 | 7.5 |
| **NanoMIP 2** |  |  |  |
| *Kd in nM (Chi2)* | *0.74(3.3)* | *110(1.7)* | *2.96(3.2)* |
| *Cross-reactivity* | *82.2%* | *0.7%* | *25%* |
| **NanoMIP 3** |  |  |  |
| *Kd in nM (Chi2)* | *0.9(1.6)* | *1300(0.8)* | *12.6(1.6)* |
| *Cross-reactivity* | *100%* | *0.07%* | *7%* |

* 1. **Electrochemical characterisation of sensor**

The insulin sensor was fabricated by immobilizing nanoMIP on the electrode. The sensor response was directly produced by the nanoMIP, which is tagged with a redox probe (ferrocene) and combines both recognition and reporting functions. Thus, the DPV current sensor response was directly related to the analyte recognition (**Figure S7**). The nanoMIP immobilisation on SPPE was confirmed by cyclic voltammetry (CV) as shown in **Figure S7**. The voltammogram displayed two characteristic peaks originated from the reversible ferrocene redox process in the nanoparticles. Insulin oxidation at SPPE is not observed in this potential range (-0.2 to 0.6 vs Ag/AgCl) as shown in **Figure S7**.

* 1. **Optimization of the sensor performance**

The sensor fabrication was improved by optimizing the immobilisation process of nanoparticles on the SPPE. For that purpose, chemical immobilisation (involving APTES and glutaraldehyde), incubation time and concentration of nanoparticles was optimized. The resulting sensors were evaluated by measuring the current response to different insulin concentrations.The concentration of APTES (1, 6 and 10%) in solution and incubation time (1 and 24 h) was optimized as shown in **Figure S8.** Short APTES incubation times (1 h) lead to low response, and high concentration of APTES resulted in aggregation and non-linear responses. The sensitivity of the sensor was increased two-fold when the chemical immobilisation of particles was settled at 6% APTES and 1 h incubation. The concentration of glutaraldehyde affected the immobilisation of the particles. The maximum sensitivity was achieved at 7% of glutaraldehyde (**Figure S9**). In addition, the sensor response was evaluated at different concentrations of nanoMIP ranged from 0.1 to 1 mg mL-1. As result, the optimal nanoMIP concentration was found at 0.5 mg mL-1, higher concentrations resulted in aggregations and drop of signal (**Figure S10A**). Furthermore, nanoMIP immobilisation time (1 to 24 h) does not affected significantly the sensor performance as shown in **Figure S10B**. The parameters optimized and the sensor performance is summarized in **Table S4**.

* 1. **Comparison of different nanoMIP compositions**

Sensors were prepared using three different nanoMIP formulations and tested against a series of insulin concentrations (50 – 2000 pM) in 5 mM PBS. The voltammetric sensor responses increased was directly proportional to the insulin solutions as shown in **Figure 1**.



**Figure 1.** (A) Sensor response to insulin prepared by using a) nanoMIP 1, b) nanoMIP 2 and c) nanoMIP 3. (B) DPV response from nanoMIP 3 for insulin concentrations at (a) 0 , (b) 50, (c) 100, (d) 200, (e) 400, (f) 600, (g) 800, (h) 1000, (i) 1200, (j) 1400, (k) 1600, (l) 1800 and (m) 2000 pM in 5 mM PBS buffer (pH = 7.4). Measurements were performed by drop casting the sample after 3 min incubation, 3 repeats per concentration.

The nanoMIP sensor presented current changes triggered by the insulin molecular recognition. The recognition prompted changes in the polymer conformation affecting the electron transfer of the ferrocene moieties, which are responsible for the overall observed electrochemical processes. Indeed, the ferrocene oxidation peak was observed at 0.3 V (vs Ag/AgCl) as shown in **Figure 1**. This increment of signal was directly correlated to the insulin concentration. Insulin electro-oxidation is not observed since that process occurs at higher potentials (+0.7 V vs Ag/AgCl). The sensors performance is summarized in **Table S5**, those results agree with the SPR results. The highest sensitivity was found for nanoMIP 3 at (1.2 ± 0.01) nA pM -1 with linear response (*R2*= 0.998) and LOD at 26 fM. Also, it was observed that insulin concentrations higher than 2200 pM produced saturation of the signal. Presumably, the saturation is caused due to the protein aggregation.

* 1. **Cross-reactivity of the sensor**

The selectivity of the insulin sensor was studied by measuring the response against interferents present in human blood such as Haemoglobin (Hb), human serum albumin (HSA) and HPC. The sensor displayed negligible response to interferents when compared to the insulin response as shown in **Figure 2** and **Table S6**. Additionally, insulin measurements were performed as well in presences of interferents. The cross-reactivity was found for HSA (16%), Hb (1.7%) and HPC (22%), demonstrating that Insulin measurements can be performed accurately in presence of potential interferents.



**Figure 2**. (A) Sensor response for (a) insulin, (b) HB, (c) HSA and (d)HPC in a concentration range from 100 to 2000 pM in 5 mM PBS.(B) Insulin measurements performed in presence of 200 pM of interferent.

* 1. **Analysis of plasma samples**

Determination of insulin concentration in human plasma was performed in the same conditions adopted for standard solutions. The sensitivity of the sensor in plasma was found at (0.74 ± 0.02) nA pM -1 with linear response (*R2*= 0.994) and LOD at 81 fM. Also, the matrix effect was estimated at 38.3% as shown in **Figure 3**. Thus, it was demonstrated that the insulin sensor can be successfully used for insulin monitoring in plasma.

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**Figure 3.** (A)Calibration plot of the insulin sensor in a) buffer and b) plasma. (B) DPV response in plasma for concentrations of insulin at (a) 0 , (b) 50, (c) 100, (d) 200, (e) 400, (f) 600, (g) 800, (h) 1000, (i) 1200, (j) 1400, (k) 1600, (l) 1800 and (m) 2000 pM . Measurements were performed by drop casting the sample after 3 min incubation, 3 replicates per concentration.

* 1. **The repeatability, reproducibility and stability of the sensor**

The reproducibility was assessed by comparing the response of five sensors to an insulin standard solution (400 pM), the relative standard deviation (RSD) value obtained was 5.6% under identical conditions. Additionally, the repeatability of the sensor was studied by measuring five times the response to the insulin standard in a single sensor, resulting in a RSD value of 4.2% under same conditions. This analysis demonstrates that the performance is compliance with the industrial accuracy standards (ISO-151917), which states that 95% of results are within ±15% of a laboratory standard. To investigate the stability of the sensor, seven sensors were prepared separately under similar conditions and stored at 4 °C for 168 days. The current response of each sensor was measured using an insulin standard in seven days intervals. The sensor response dropped to 35% after this period as shown in **Figure S11**.

* 1. **Comparison between sensors for insulin detection**

Different transducers and fabrication strategies have been proposed to monitor insulin levels in human plasma (**Table 2**). Some of them include the combination of metal and carbon nanostructures to increase the electro-catalytic activity for electro-oxidation of insulin. (Habibi et al. 2016; Lin et al. 2014; Martínez-Periñán et al. 2016; Yagati et al. 2016)Unfortunately these methods face low selectivity and a lack of sufficient sensitivity. Alternatively, affinity sensors (non-oxidative methods) involving aptamers, antibodies and MIP display inherent selectivity for a given target.(Ensafi et al. 2017; Kartal et al. 2019; Luo et al. 2013; Zhu et al. 2016)

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| **Table 2**. Performance of insulin electrochemical sensor in human plasma |
| *Sensor* | *Detection**method* | *Concentration* *range (pM)* | *LOD (pM)* | *Sensitivity* | *RSD**(%)* | *Stability* *(days)* | *Recovery* *(%)* | *Ref.* |
|  MIP/AuE | DPV | 0.01 – 0.5 | 7.24x10-3 | -121.5 µA pM-1 | 1.41 | 14 | 94.5-104.1 | (Cheng-Jun et al. 2017) |
| MWCNT/MIP | DPASV | 68 – 5682 | 18 | 1.6 µAnM-1 | 0.87 | 25 | 99.3-101.4 | (Prasad et al. 2010) |
| Ni(OH)2NP/Nafion-MWCNT/GCE | CV | 1.5×106 – 40×106 | 8.5 x 104 | 1.1 A mol cm-2 μM-1 | 2.7 | 60 | 80 ± 6 | (Martínez-Periñán et al. 2016) |
| Ni(OH)2-GN/GCE | CA | 8 x 105 – 64 x 105 | 2 x 105 | 0.05 µA µM-1 | N.R. | N.R. | 92.5-105.3 | (Lin et al. 2014) |
| AgNP-rGO | EIS | 1.7×102 – 1.7×105 | 1.2 x 10-2 | 0.53 kΩng mL-1 | <5.7 | 7 | 91.8-102.4 | (Yagati et al. 2016) |
| Co(OH)2NP/CCE | CV | 500 – 1.5×104 | 110 | 11.8 nA nM-1 | 2.3 | 70 | 95-104 | (Habibi et al. 2016) |
| Aptamer/AuNP/GE | EIS | 1x103 – 1 x 106 | 270 | 0.1327 Ω nM-1 | 5.1 | 10 | 88.9-103 | (Ensafi et al. 2017) |
| Immuno-sensor | EIS | 0.1 – 200  | 4.26 x 10-2 | N.R. | 5 | 30 | N.R. | (Luo et al. 2013) |
| MIP/GCE | DPV | 10 – 1000 | 3 | - 16.09 µA nM-1 | 4.8 | 20 | N.R. | (Zhu et al. 2016) |
| GCE/rGO | CA | 4×104 – 64×104  | 350 | 7.12 nA nM-1 | 6.1 | 5 | 97–105 | (Noorbakhsh and Alnajar 2016) |
| Immuno-sensor | CA | 1.72x 10-2 – 8608 | 4.3x 10-5 | 7.6 μA ng-1mL-1 | 4.8 | 14 | 99.3-102.7 | (Li et al. 2018) |
| NanoMIP/SPPE | DPV | 50-2000  | 0.081 | 0.74 nA pM-1 | 4.2 | 168 | 99.5-101 | This work |
| The main advantages (a)selectivity, (b)robustness, (c) easy integration protocols,(d) applicability in relevant clinical ranges |
| Nanoparticles (NP), Glassy-carbon electrodes (GCE), multi-walled carbon nanotubes (MWCNT), graphite electrode (GE), Gold electrode(AuE), reduced graphene oxide (rGO), carbon ceramic electrode(CCE), carbon paste electrode (CPE),electrochemical impedance spectroscopy (EIS) chronoamperometry (CA), differential pulse anodic stripping voltammetry (DPASV). |

Classically, response is measured indirectly using a redox probe in solution by voltammetric or impedimetric techniques. These methods are more selective, however bio-sensors require a costly recognition element (aptamers, antibodies and enzymes) and tedious preparation, with an additional lack of stability and short shelf life. Alternatively, MIP can be used as catalytic or affinity sensors, resulting in more stable systems.(Cheng-Jun et al. 2017; Zhu et al. 2016) To increase the surface area and improve the sensitive of the recognition elements, nanostructures such as carbon nanotubes and graphene have been used.(Martínez-Periñán et al. 2016; Prasad et al. 2010) Nevertheless, there is a lack of compatibility between the fabrication process and the complexity of those systems, resulting in a sensor technology that cannot be industrially transferred or scale up. Consequently, these systems cannot be applied in point of care scenarios limiting their Technology Readiness Levels (TRL) progress.

To overcome those problems the present sensor technology uses electroactive nanoMIP, which confers several advantages when compared to previous biosensor and MIP technology. For instance, nanoMIP allow the recognition of target epitopes, which can be precisely evaluated computationally, as result, the experimental selectivity is enhanced. Besides, this electro-activity of nanoMIP confers direct recognition and reporting capabilities leading to a higher sensitivity and low cross-reactivity in biological samples. The main benefits of the present sensor technology are: (a) their robustness, originating from polymeric nature of nanoMIP, (b) easy integration protocols, and compatibility with mass production e.g. printing, and (c) generic nature and applicability to broad range of targets. Potentially, nanoMIP-based sensors can have a revolutionary impact in clinical point-of-care diagnostics, as well as environmental, defence and food monitoring applications.

1. **Conclusion**

The electro-responsive nanoMIP technology allow the computationally design and production of reliable and robust sensor devices for highly sensitive and selective recognition of insulin for clinical applications. The present sensor design offers simple integration system and straight forward fabrication, without the need of biological components. The synthetic preparation of nanoMIP offers the advantage of relative low cost and capability of mass production using industrial printing techniques. The sensor displayed low cross-reactivity against structural interferents, confirming its suitability for relevant clinical quantification of insulin in human plasma samples. The present work is a step forward in directions of developing highly robust sensor, compatible with mass industrial production and that can be operated in real scenarios as near patient test by relatively unexperienced personnel. Future work will intend to bring this sensor for testing in hospital environment. Furthermore, the developed nanoMIP sensor could also be used for measuring insulin in pharmaceutical formulations for routine quality control applications.

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**References**

Ahmad, O.S., Bedwell, T.S., Esen, C., Garcia-Cruz, A., Piletsky, S.A., 2019.Trends in biotechnology 37(3), 294-309.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. *Nucleic Acids Research*,28(1), 235-242.

Blum, W.F., Ranke, M.B., Bierich, J.R., 1988. European Journal of Endocrinology 118(3), 374-380.

Bollella, P., Fusco, G., Tortolini, C., Sanzò, G., Favero, G., Gorton, L., Antiochia, R., 2017. Biosensors and Bioelectronics 89, 152-166.

Cowen, T., Karim, K., Piletsky, S., 2016. Analytica chimica acta 936, 62-74.

Chen, Z., Caulfield, M.P., McPhaul, M.J., Reitz, R.E., Taylor, S.W., Clarke, N.J., 2013. Clinical chemistry 59(9), 1349-1356.

Cheng-Jun, Z., Xiong-Hui, M., Jian-Ping, L., 2017. Chinese Journal of Analytical Chemistry 45(9), 1360-1366.

Dayaldasani, A., Rodriguez Espinosa, M., Ocon Sanchez, P., Perez Valero, V., 2015. Annals of clinical biochemistry 52(3), 312-318.

Ensafi, A.A., Khoddami, E., Rezaei, B., 2017. Colloids and Surfaces B: Biointerfaces 159, 47-53.

Fu, Z., R Gilbert, E., Liu, D., 2013. Current diabetes reviews 9(1), 25-53.

Garcia-Cruz, A., Cowen, T., Voorhaar, A., Piletska, E., Piletsky, S.A., 2020. The Analyst, 2020,145, 4224-4232.

Gaster, R.S., Hall, D.A., Nielsen, C.H., Osterfeld, S.J., Yu, H., Mach, K.E., Wilson, R.J., Murmann, B., Liao, J.C., Gambhir, S.S., 2009.. Nature medicine 15(11), 1327.

Habibi, E., Omidinia, E., Heidari, H., Fazli, M., 2016. Analytical biochemistry 495, 37-41.

Hovancová, J., Šišoláková, I., Oriňaková, R., Oriňak, A., 2017. Journal of Solid State Electrochemistry 21(8), 2147-2166.

Kartal, F., Çimen, D., Bereli, N., Denizli, A., 2019. Materials Science and Engineering: C 97, 730-737.

Leach, A.R., 2001. Pearson education.

Li, Y., Tian, L., Liu, L., Khan, M.S., Zhao, G., Fan, D., Cao, W., Wei, Q., 2018. Talanta 179, 420-425.

Lin, Y., Hu, L., Li, L., Wang, K., 2014. RSC Advances 4(86), 46208-46213.

Luo, X., Xu, M., Freeman, C., James, T., Davis, J.J., 2013. Analytical chemistry 85(8), 4129-4134.

Martínez-Periñán, E., Revenga-Parra, M., Gennari, M., Pariente, F., Mas-Ballesté, R., Zamora, F., Lorenzo, E., 2016. Sensors and Actuators B: Chemical 222, 331-338.

McPherson, R.A., Pincus, M.R., 2017. Henry's Clinical Diagnosis and Management by Laboratory Methods E-Book. Elsevier Health Sciences.

Nicol, D., Smith, L., 1960. Nature 187(4736), 483.

Noorbakhsh, A., Alnajar, A.I.K., 2016. Microchemical Journal 129, 310-317.

Piletska, E.V., Guerreiro, A., Mersiyanova, M., Cowen, T., Canfarotta, F., Piletsky, S., Karim, K., Piletsky, S., 2019. Langmuir 36(1), 279-283.

Piletsky, S.A., Karim, K., Piletska, E.V., Day, C.J., Freebairn, K.W., Legge, C., Turner, A.P.F., 2001. Analyst 126(10), 1826-1830.

Prasad, B.B., Madhuri, R., Tiwari, M.P., Sharma, P.S., 2010. Electrochimica Acta 55(28), 9146-9156.

Sabu, C., Henna, T., Raphey, V., Nivitha, K., Pramod, K., 2019. Advanced biosensors for glucose and insulin. Biosensors and Bioelectronics.

Sanger, F., 1959. Chemistry of insulin. Science 129(3359), 1340-1344.

Santen, R.J., Willis, P.W., Fajans, S.S., 1972. Archives of internal medicine 130(6), 833-843.

Sapin, R., 2002. Clinical chemistry and laboratory medicine 40(7), 705-708.

Schirhagl, R., Latif, U., Podlipna, D., Blumenstock, H., Dickert, F.L., 2012. Analytical chemistry 84(9), 3908-3913.

Schirhagl, R., Podlipna, D., Lieberzeit, P.A., Dickert, F.L., 2010. Chemical communications 46(18), 3128-3130.

Selvolini, G., Marrazza, G., 2017. Sensors 17(4), 718.

Shen, Y., Prinyawiwatkul, W., Xu, Z., 2019. Analyst, 144(14), 4139-4148.

Šišoláková, I., Hovancová, J., Oriňaková, R., Oriňak, A., Trnková, L., Třísková, I., Farka, Z., Pastucha, M., Radoňák, J., 2020. Journal of Electroanalytical Chemistry 860, 113881.

Staten, M.A., Stern, M.P., Miller, W.G., Steffes, M.W., Campbell, S.E., Workgroup, I.S., 2010. Diabetes care 33(1), 205-206.

Timofeev, V.I., Chuprov-Netochin, R.N., Samigina, V.R., Bezuglov, V.V., Miroshnikov, K.A., Kuranova, I.P., 2010. Acta Crystallogr Sect F Struct Biol Cryst Commun 66(Pt 3), 259-263.

Verhaeghe, J., Van Bree, R., Van Herck, E., Laureys, J., Bouillon, R., Van Assche, F.A., 1993. American journal of obstetrics and gynecology 169(1), 89-97.

Warnken, T., Huber, K., Feige, K., 2016. BMC veterinary research 12(1), 196.

Wilcox, G., 2005. Clinical biochemist reviews 26(2), 19.

Williamson, M.A., Snyder, L.M., 2014. Wallach's Interpretation of Diagnostic Tests: Pathways to Arriving at a Clinical Diagnosis. Lippincott Williams & Wilkins.

Yagati, A.K., Choi, Y., Park, J., Choi, J.-W., Jun, H.-S., Cho, S., 2016. Biosensors and Bioelectronics 80, 307-314.

Zhu, W., Xu, L., Zhu, C., Li, B., Xiao, H., Jiang, H., Zhou, X., 2016. Electrochimica Acta 218, 91-100.