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### **► To cite this version:**

Ivan S Kucherenko, Carole Farre, Gaetan Raimondi, Carole Chaix, Nicole Jaffrezic-Renault, et al.. A novel adenosine triphosphate (ATP) biosensor based on electrospun polymer nanofibers with entrapped hexokinase and glucose oxidase. *Applied Nanoscience*, 2023, 13 (10), pp.7037 - 7045. 10.1007/s13204-023-02861-y . hal-04814644

**HAL Id: hal-04814644**

**<https://hal.science/hal-04814644v1>**

Submitted on 2 Dec 2024

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# **A novel adenosine triphosphate (ATP) biosensor based on electrospun polymer nanofibers with entrapped hexokinase and glucose oxidase**

Ivan S. Kucherenko<sup>1,2,\*</sup>, Carole Farre<sup>3</sup>, Gaetan Raimondi<sup>3</sup>, Carole Chaix<sup>3</sup>, Nicole Jaffrezic-Renault<sup>3</sup>, Jean-François Chateaux<sup>4</sup>, Maksim Sobolevskyi<sup>1</sup>, Oleksandr O. Soldatkin<sup>1</sup>, Sergei V. Dzyadevych<sup>1,5</sup>, Alexei P. Soldatkin<sup>1,5</sup>, Florence Lagarde<sup>3</sup>

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## **Abstract**

In this work, a novel amperometric biosensor for the determination of adenosine triphosphate (ATP) was developed. The biosensor was based on two enzymes (glucose oxidase and hexokinase) that were entrapped in the electrospun nanofibers. The biosensor was prepared by direct electrospinning of the enzymes/polymer mixture on the surface of platinum disc electrodes with subsequent UV cross-linking of the obtained nanofibers. Photocrosslinkable polymer (polyvinyl alcohol containing styrylpyridinium groups, PVA-SbQ) was chosen as matrix, while gold nanoparticles (GNPs) were added to the enzymes/polymer mixture to improve analytical characteristics of the biosensor. For the first time, incorporation of several enzymes in the nanofibers during electrospinning was demonstrated. Composition of the mixture for electrospinning was optimized. The obtained biosensors enabled successful detection of ATP by chronoamperometry, linear range was 25-200  $\mu\text{M}$ . The analytical characteristics of the biosensors were compared with the biosensors prepared by manual drop-casting of the enzymes/polymer/GNP mixture. The biosensor can be also used to detect glucose in samples with known or very low ( $<10 \mu\text{M}$ ) ATP concentration; linear range of the glucose determination was 0.01-3 mM. The described approach for one-step electrospinning of several enzymes is fast and simple and can be easily modified for immobilization of other multi-enzyme systems, which

opens possibilities to enlarge the range of the targets that can be determined by the biosensors produced using electrospinning.

**Keywords:** Electrospun nanofibers; Gold nanoparticles; Glucose oxidase; Hexokinase; Adenosine triphosphate; Electrochemical biosensor.

## **Introduction**

Electrochemical biosensors are analytical devices used for the detection of substances in various mediums, such as water-based solutions. They are a subclass of chemical sensors and consist of a receptor element connected to an electrochemical transducer (either amperometric, conductometric, potentiometric, or some other type) (Ronkainen et al. 2010). Nowadays immobilized enzymes are often used as receptors of the electrochemical biosensors since they are selective, reusable, and their catalysis rate is very fast (Das et al. 2016).

Process of enzyme immobilization on the transducer's surface strongly affects the enzyme activity and stability that, in turn, has great effect on the biosensor characteristics. Thus, the biosensor performance strongly depends on the immobilization conditions, such as properties of supports, and composition of immobilization mixture (Sassolas et al. 2012; Soldatkina et al. 2019).

Electrospinning is a quite simple method for producing large amounts of long and uniform nanofibers. The nanofibers can be diverse in composition and have different chemical and morphological modifications as well as large surface area to volume ratio. Due to these features, usage of electrospun nanofibers is studied for various biomedical, electronic and industrial applications (Bhardwaj and Kundu 2010; Chigome and Torto 2011; Thenmozhi et al. 2017). Particularly, enzyme immobilization using electrospun nanofibers receives great attention (Wang et al. 2009). The enzymes can be either added to the initial mixture before electrospinning with subsequent entrapment inside the fibers or attached to the fibers after electrospinning. Entrapment is easier since it requires fewer steps for the biosensor preparation. However, in case of entrapment some enzyme molecules will be located deeply inside the fibers with no contact with the surface. Dense polymer network may significantly limit diffusion of molecules to enzymes, thus reducing amount of active enzyme molecules on the biosensor's surface. On the other hand, usually enzymes are not strongly bound to polymers and can escape from fibers if polymer density is too low. For these reasons, entrapment is not always feasible. PVA-SbQ is one of polymers which are capable of entrapping enzymes while leaving enough space for small molecules to diffuse through the polymer network (RoyChoudhury et al. 2018). It was shown earlier that 20  $\mu\text{m}$  and 30  $\mu\text{m}$  thick PVA-SbQ films allow sufficient diffusion of small molecules

for functioning of entrapped enzymes; thus, this polymer is not expected to cause diffusion issues in form of nanofibers (Marty et al. 1992; Braiek et al. 2018).

In recent years, a number of biosensors that are based on enzymes and electrospun nanofibers were developed. In most cases, the biosensors were prepared by two- or three-stage procedure, which included synthesis of nanofibers, possible treatment and activation of the fibers and the enzyme immobilization onto the fibers. The enzymes were immobilized either by physical adsorption or covalent binding (mostly via glutaraldehyde); the enzymes include acetylcholinesterase (detection of pirimiphos-methyl) (El-Moghazy et al. 2016), cholesterol oxidase (cholesterol) (Shin and Kameoka 2012) fructose dehydrogenase (fructose) (Marx et al. 2011), glucose oxidase (glucose) (Manesh et al. 2008; Demirci Uzun et al. 2014), alcohol oxidase (ethanol) (Zhao et al. 2013), papain (metal ions) (Moreno-Cortez et al. 2015), uricase (uric acid) (Numnuam et al. 2014), laccase (catechol) (Li et al. 2014). A method which seems to be more favorable consists of one-step biosensor preparation, when the enzymes are mixed with the polymer and this mixture is being electrospun. Thus, the resulting nanofibers entrap enzymes and there are no additional immobilization steps. However, only a few works described biosensors based on entrapment of glucose oxidase (detection of glucose) (Ren et al. 2006; Su et al. 2013; Sapountzi et al. 2015, 2017) and hemoglobin (hydrogen peroxide) (Guo et al. 2011).

All the reported biosensors created with electrospinning contain only one enzyme in the recognition elements. This is a significant omission that limits number of substances, which can be detected by the biosensors. Thus, a perspective approach is usage of multi-enzyme systems, in which enzymes are catalyzing sequential reactions, resulting in creation of a detectable product. Immobilization of multi-enzyme systems gives possibility to expand the range of detectable substances by indirect detection of enzymatic reactions, which do not generate electrochemical signal (Kirsch et al. 2013). However, biosensors incorporating electrospun nanofibers with multi-enzyme systems have not been reported yet.

Adenosine triphosphate (ATP) is an important biological substance that plays a key role in energy metabolism. Moreover, ATP acts as a signaling molecule involved in immune response and other processes; its role is extensively studied nowadays (Lombardi et al. 2021; Pinna et al. 2022). Several ATP-sensitive biosensors were described recently. They include a fluorescent biosensor based on aptamers and covalent organic framework for the detection of ATP and trombine (Jouha et al. 2023), DNAzyme-based ATP biosensors with colorimetric or fluorescent detection (Cai et al. 2023; Si et al. 2023), and biosensors based on glucose oxidase (GOx) and hexokinase (HEX) (Zhu et al. 2020; Lu et al. 2023). In general, biosensors based on nucleic acids demonstrate higher sensitivity, but they are disposable or have a complex procedure for reuse. On the other hand, enzyme-based biosensors can be easily used for repetitive ATP

measurements; another advantage is the possibility of real-time detection, which is important for biomedical applications.

In this work, we propose for the first time an amperometric ATP-sensitive biosensor based on two enzymes – GOx and HEX, which were co-immobilized in electrospun PVA-SbQ nanofibers. Electrospinning conditions and characteristics of the obtained biosensors were investigated.

## **Materials and methods**

### *Materials*

Hexokinase (HEX, from *Saccharomyces cerevisiae*, with activity  $\geq 25$  U/mg), glucose oxidase (GOx, from *Aspergillus niger*, with activity 128 U/mg), bovine serum albumin (BSA, fraction V,  $\geq 96\%$ ), glucose ( $\geq 99.5\%$ ), adenosine triphosphate (ATP, disodium salt hydrate, grade 1,  $\geq 99\%$ ), magnesium chloride ( $\geq 99\%$ ), glycerol ( $\geq 99\%$ ), HEPES ( $\geq 99.5\%$ ), polyvinyl alcohol (PVA, 87-89% hydrolyzed, Mw 85000-124000), polyethylenimine (PEI, 50% aqueous solution, Mw  $\sim 750000$ , Mn  $\sim 60000$ ), and glutaraldehyde (GA, 50% aqueous solution) were produced by Sigma-Aldrich (USA). Polyvinyl alcohol bearing styrylpyridinium pendent groups (PVA-SbQ, SPP-H-13: degree of polymerization 1700, degree of saponification 88, betaine SbQ 1.3 mol%, solid content 13.35 wt%, pH 6.2) was from Toyo Gosei (Japan). Ultrapure water with resistivity  $> 18$  M $\Omega$ -cm from a MilliQ purifier was used.

Gold nanoparticles (GNPs) (18 nm average size,  $1.1 \times 10^9$  NPs/mL) were synthesized according to the earlier described procedure (Sapountzi et al. 2017).

### *Design of amperometric transducers*

Platinum disc electrodes were used as transducers. Sensitive area of the electrodes contained a platinum wire (0.4 mm in diameter) sealed at the end of a glass capillary. More details about the electrodes can be found in our earlier works (Kucherenko et al. 2017). Platinum serves as catalyst for hydrogen peroxide oxidation, and for this reason it is widely used for creation of biosensors which are based on H<sub>2</sub>O<sub>2</sub>-producing enzymes, including GOx. It is possible to reduce sensitivity of the electrode to interfering electroactive compounds (i.e., ascorbic acid) by covering the electrode surface with nanosized phenylenediamine-based membranes (Soldatkina et al. 2017; Pyeshkova et al. 2022).

The electrodes were reusable; surface of the electrodes was cleaned between experiments using ethanol wetted cotton swab. Furthermore, the surface was polished with sandpaper and alumina powder (particles of 0.1  $\mu\text{m}$  and 0.05  $\mu\text{m}$ ).

### *Immobilization of the enzymes by drop-casting*

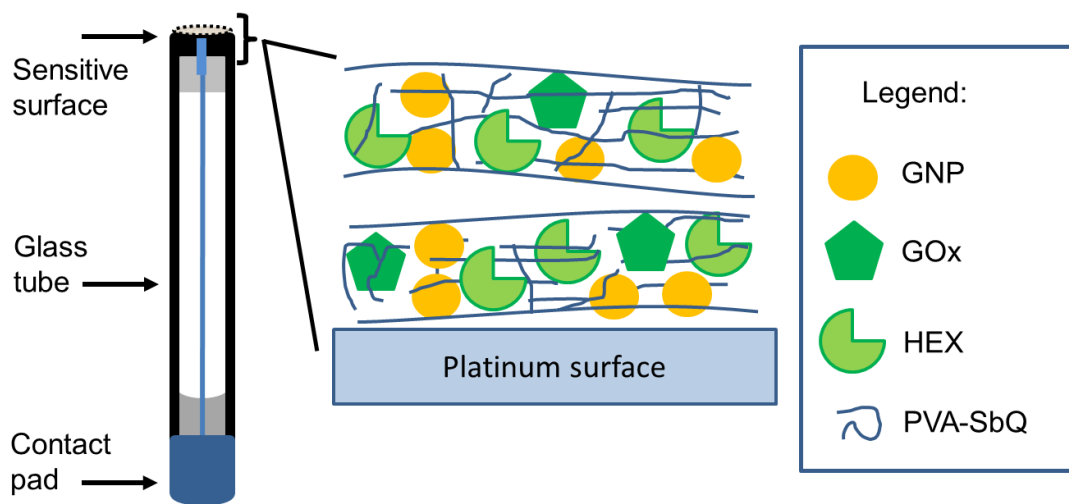
Procedures of GOx/HEX immobilization by drop-casting were described in detail earlier (Dridi et al. 2015; Kucherenko et al. 2017). Three immobilization methods were used: enzyme crosslinking by GA, entrapment in PVA-SbQ matrix, and entrapment in PVA/PEI matrix. The general idea of all immobilization methods was to have the same concentration of GOx (0.5%, or 0.096 U per biosensor) and HEX (2.5%, or 0.094 U per biosensor) in all bioselective elements of the biosensors in order to compare effectiveness of different approaches.

### *Electrospinning procedure*

Nanofibers were fabricated using an electrospinning device of our own production (Sapountzi et al. 2015). The spinneret was made of a 5 ml glass syringe (Fortuna Optima, Germany) equipped with a Luer lock port to connect blunt-end needles (22 x 1-1/2 inches, Cadence Science, USA).

For each electrospinning procedure, fresh mixture (0.5% GOx, 2.5% HEX, 6.67% PVA-SbQ, with or without of  $5.5 \times 10^8$  GNPs/mL) was prepared. The enzymes were stirred with the polymer during 30 min in a beaker and then placed into a syringe. Volume of the mixture was about 1 ml for the convenience of operation. To perform electrospinning, we placed one clean working electrode in the ES setup and connected it to the ground electrode of the power supply. Then high voltage (11.4 kV) was applied between the syringe needle and the ground, pressure (0.58 bar) was applied to the syringe jack, and nanofibers were generated. Simultaneous coverage of 2 and 3 electrodes was also tested, but resulting nanofibers mats were not uniform, so we preferred to cover the electrodes one-by-one.

Afterwards the transducers with deposited nanofibers were placed for 10 min at in a Bio-link BLX UV-cross-linker (Vilbert Lourmat) equipped with one 100 W UV lamp (365 nm). This procedure was the same as for usual PVA-SbQ immobilization and was needed for polymer photo cross-linking. Scheme of the obtained biosensors is represented in the Fig. 1.



**Fig. 1** Scheme of the ATP biosensor based on GOx/HEX/PVA-SbQ/GNPs nanofibers

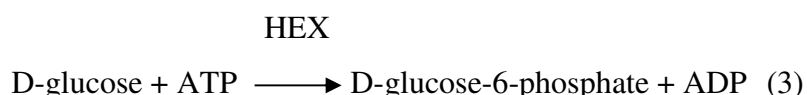
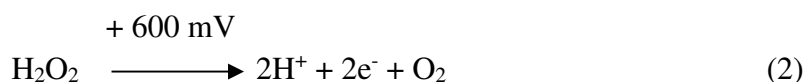
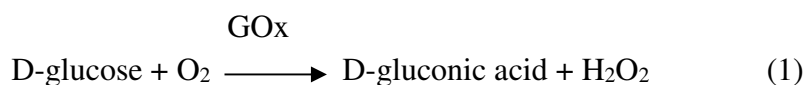
### *Working conditions*

Typical three-electrode setup was used. Platinum disc transducer was used as a working electrode, platinum wire – as a counter electrode, and saturated Ag/AgCl electrode (Hach Lange, France) was a reference electrode. All the electrodes were placed in an open cell, at permanent stirring by a magnetic stirrer and at constant potential of 0.6 V versus the reference electrode. Computer-controlled PalmSens potentiostat (Palm Instruments BV, Netherlands) was used for chronoamperometric measurements. 10 mM HEPES, pH 7.4, with 3 mM of  $Mg^{2+}$  was used as the working buffer.

## **Results and discussion**

### *The principle of biosensor operation*

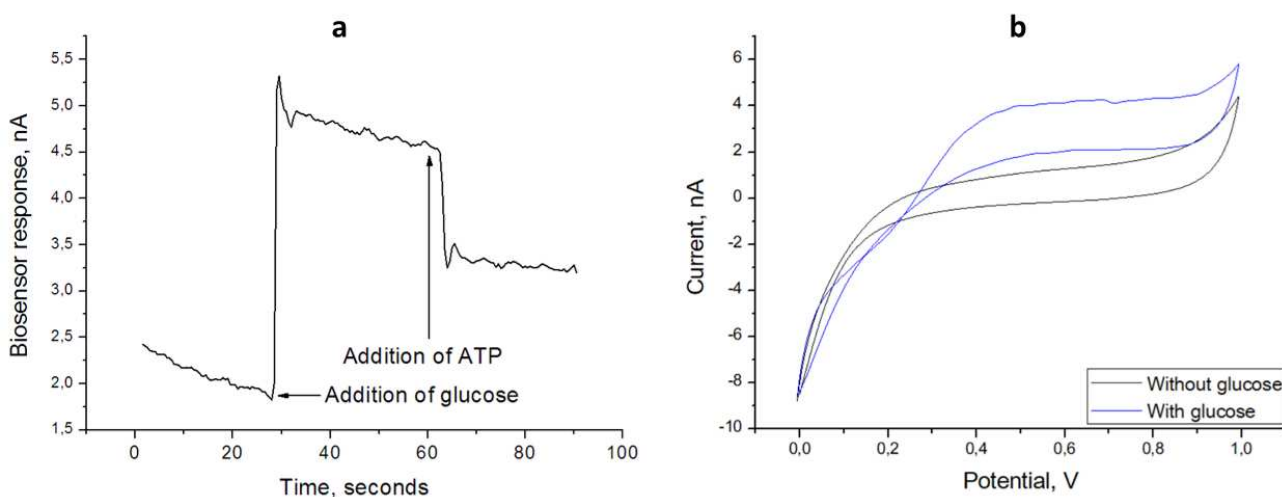
The biosensor operation is based on three reactions, which are occurring on the biosensor's surface:



GOx and HEX compete for glucose. In the absence of ATP, GOx reaction takes place at full capacity, leading to  $\text{H}_2\text{O}_2$  production. The latter is detected by chronoamperometry. In the

presence of ATP, HEX phosphorylates glucose, decreasing glucose concentration and decreasing H<sub>2</sub>O<sub>2</sub> production. Thus, the biosensor signal is directly proportional to glucose concentration and inversely proportional to ATP concentration. It is not possible to detect concentration of both substances simultaneously. Main aim of this biosensor is to determine ATP concentration in samples that contain known concentration of glucose. Also it is possible to detect ATP in glucose-free samples by adding glucose manually. On the other hand, the biosensor can be used to determine glucose concentration in samples with no ATP or when ATP concentration is known.

An example of the biosensor response to glucose and ATP (chronoamperometry) and glucose detection using cyclic voltammetry are presented in Fig. 2. It can be seen that the response is rather fast and occurs within seconds after addition of the substances. Cyclic voltammetry shows that the biosensor can be operated in the range of potentials from 0.5 V to 0.9 V, which is determined by H<sub>2</sub>O<sub>2</sub> oxidation.



**Fig. 2** An example of the biosensor response to sequential additions of 50  $\mu\text{M}$  glucose and ATP (a) and cyclic voltammograms in the absence and in the presence of glucose (b), obtained at start potential 0 V, final potential 1 V, scan rate 20 mV/s, step of potential change 5 mV

#### *Selection of the polymer for GOx and HEX immobilization*

At the first stage of the research it was necessary to select an electrospinnable polymer, suitable for GOx/HEX immobilization. We chose PVA-SbQ and PVA/PEI, since earlier we successfully used them for creation of GOx-based biosensors using electrospinning (Sapountzi et al. 2015, 2017). To prevent dissolution of the polymers in the working buffer after polymer deposition on the electrode, PVA-SbQ was photopolymerized under UV light, while PVA/PEI was cross-linked using GA. Furthermore, cross-linking of the enzymes with BSA by GA was also tested for comparison (Compagnone and Guilbault 1997; Soldatkin et al. 2009). Immobilization was performed by drop-casting the enzyme/polymer mixtures onto the electrode

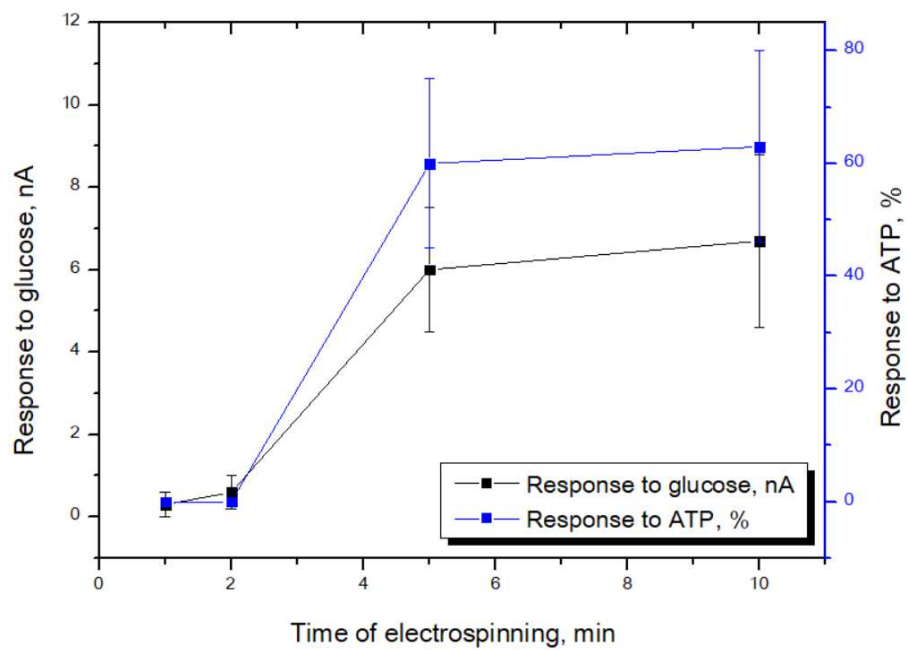


surface. As a result, we obtained dense polymer membranes with immobilized enzymes. By doing this, we wanted to check that the enzymes retain their activity inside the polymer membranes and to select polymer in which sensitivity to ATP is the highest.

It was found that the biosensors created with all types of immobilization had high sensitivity to glucose, with a linear range from 10  $\mu\text{M}$  to 3 mM. This is explained by high stability of GOx, which is a robust enzyme which retains activity after various immobilization procedures. Results obtained with all biosensors were close to each other. Situation with ATP detection was different. Usage of UV and PVA-SbQ led to the creation of biosensors with the linear range of ATP determination up to 100  $\mu\text{M}$ . On the other hand, biosensors based on PVA/PEI immobilization were not sensitive to ATP at all. Most likely, HEX interaction with highly positively charged PEI led to inactivation of the enzyme. Considering this, PVA-SbQ was chosen for the further work.

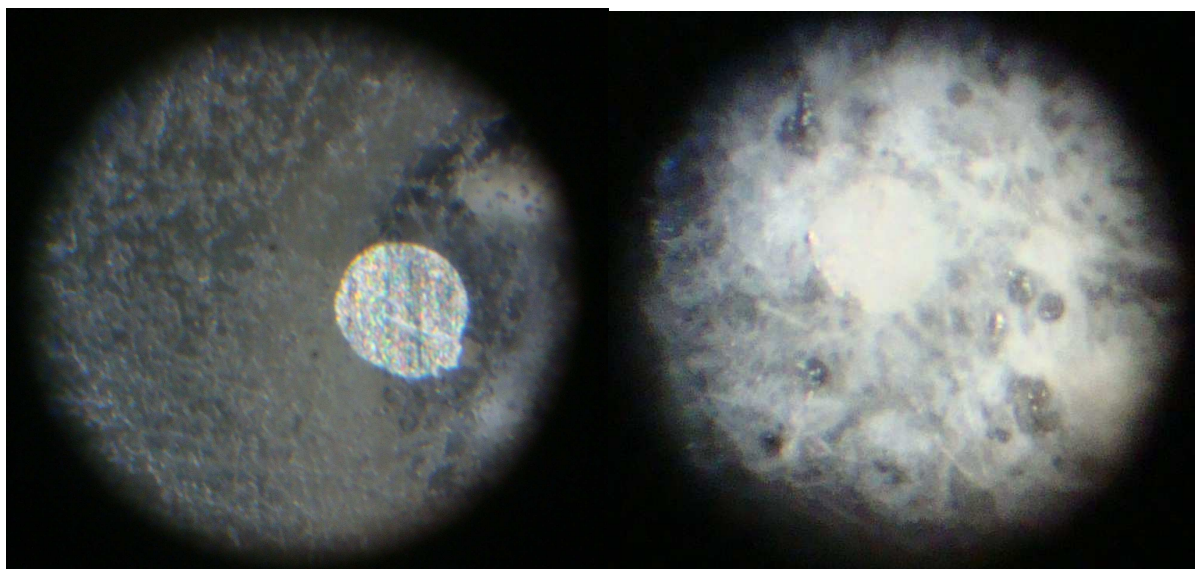
#### *Selection of the duration of electrospinning*

At the beginning of the electrospinning experiments, we used the following mixture placed into the syringe: 0.5% GOx, 2.5% HEX, 6.67% PVA-SbQ. GNPs were added in further experiments because we wanted to start electrospinning with a simpler solution. At the first step we selected an optimal duration for coverage of the amperometric transducers with enzyme-containing nanofibers. Thus, electrospinning procedure was performed during 1 to 10 min (Fig. 3). As seen, one or two minutes were not enough to obtain working biosensors, although some nanofibers were observed on the transducers surface. On the other hand, 5 min electrospinning led to the deposition of a dense nanofibers mat, and the biosensor responses were quite good. Further increase of the electrospinning duration to 10 min did not lead to significant improvement of the biosensor work, thus 5 min duration was selected as optimal and was used in later work.



**Fig. 3** Comparison of the biosensor responses to glucose and ATP obtained with biosensors covered with nanofibers during different electrospinning duration. Response to glucose (100  $\mu\text{M}$ ) was recorded in the absence of ATP. Response to ATP (100  $\mu\text{M}$ ) was recorded in the presence of 100  $\mu\text{M}$  glucose

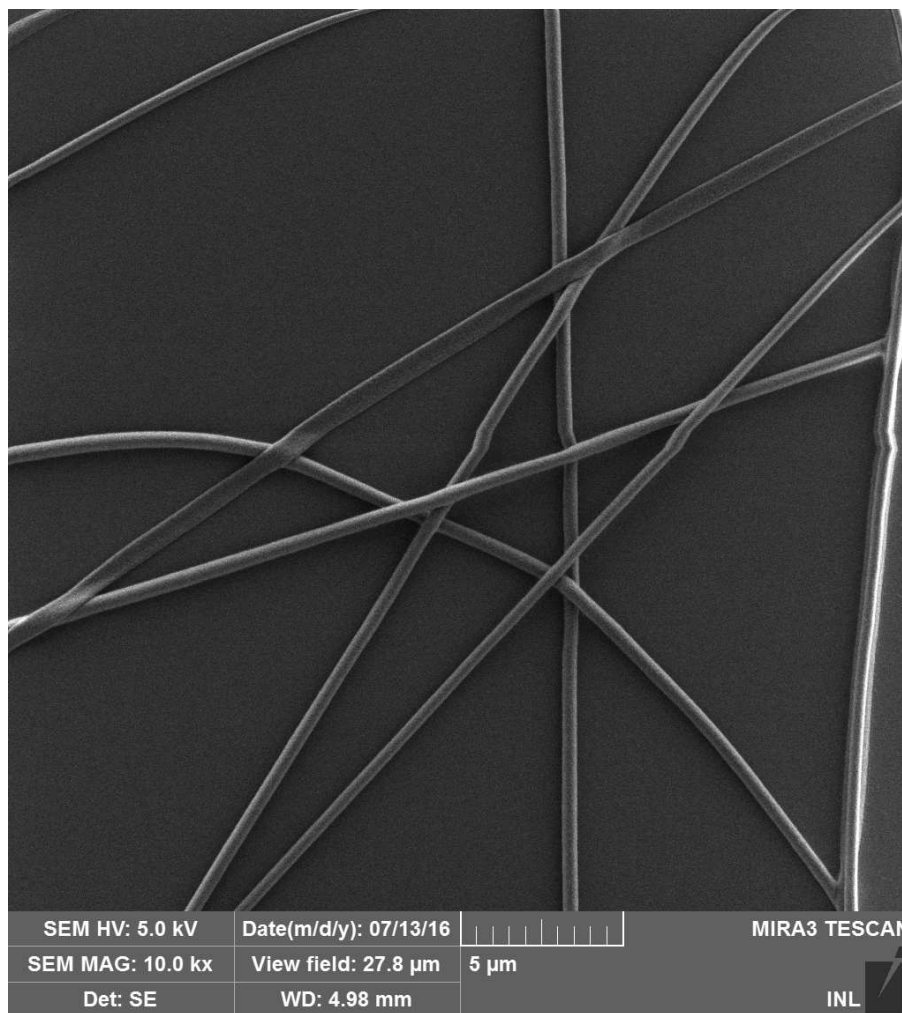
Effectiveness of the transducer coverage with nanofibers was checked with optical microscope (Fig. 4). On the images a glass surface with embedded Pt wire – a sensitive region of the transducer – can be seen. Deposition of a nanofibers mat with a random density can be observed at the right image, which suggests that electrospinning was successful.



**Fig. 4** Optical images of the amperometric transducers. Clean active region of the transducer (left) and the region after 5-min deposition of nanofibers by electrospinning (right). Diameter of the platinum disc (the brightest part on the images) is 0.4 mm

Gold nanoparticles (GNPs) are frequently used in enzyme-based biosensors. They improve stability of immobilized enzymes, participate in redox processes, and improve analytical characteristics of the biosensors (Sapountzi et al. 2017; Jiang et al. 2018). During next experiments we prepared enzymes/polymer mixtures containing  $2.8 \times 10^8$  GNPs/mL and later –  $5.5 \times 10^8$  GNPs/mL. The mixtures appeared to be quite easy for electrospinning, and nanofibers were successfully obtained. Thus, we created biosensors based on three nanofiber compositions: (1) 0.5% GO<sub>x</sub>, 2.5% HEX, 6.67% PVA-SbQ; (2) same with  $5.5 \times 10^8$  GNPs/mL; (3) same as (1) with  $5.5 \times 10^8$  GNPs/mL.

Simultaneously with electrospinning of the mixtures onto the electrodes we deposited the same nanofibers onto glass plates and metalized them for scanning electron microscopy. Obtained SEM images are presented in the Fig. 5. Average fiber diameter of the nanofibers without GNPs was  $520 \pm 48$  nm. Fibers with  $2.8 \times 10^8$  GNPs/mL had average diameter  $472 \pm 45$  nm, and with  $5.5 \times 10^8$  GNPs/mL –  $377 \pm 34$  nm. Addition of GNPs is believed to have changed electrical properties of the enzyme/polymer solution which led to the decrease of nanofiber diameter.



**Fig. 5** SEM images of the electrospun nanofibers on a glass substrate. Composition of the source solution for electrospinning: 0.5% GOx, 2.5% HEX, 6.67% PVA-SbQ, with  $2.8 \times 10^8$  GNPs/ml

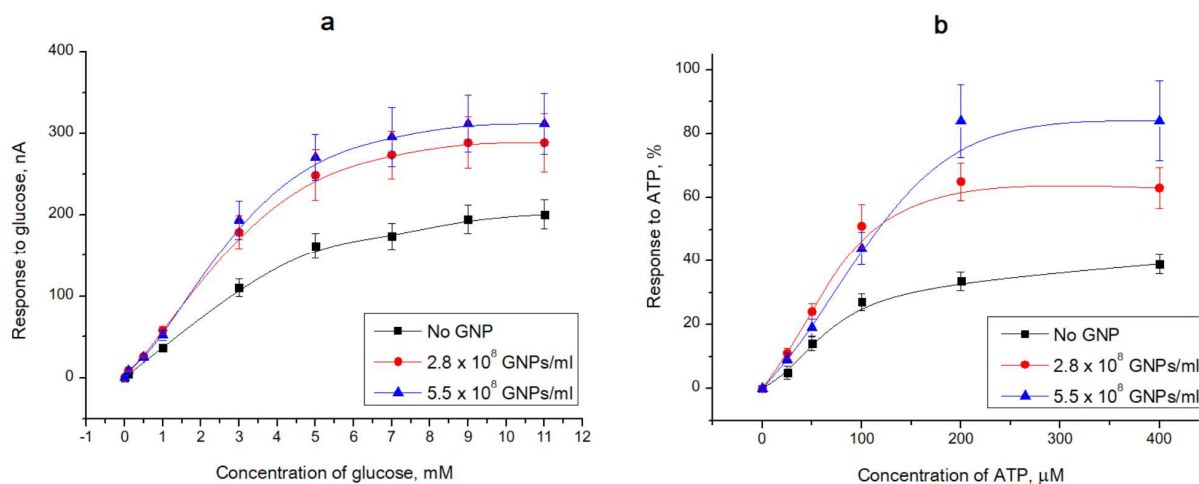
#### *Analytical characteristics of the biosensors*

Calibration curves for glucose and ATP determination were obtained (Fig. 6). The highest sensitivity to both glucose and ATP was demonstrated by the biosensor based on nanofibers with  $5.5 \times 10^8$  GNPs/mL. Biosensor with  $2.8 \times 10^8$  GNPs/mL had a bit smaller sensitivity to both substances and more narrow range of ATP determination (up to 100  $\mu$ M instead of 200  $\mu$ M). Biosensor without GNPs had the lowest sensitivity to both substrates. On the other hand, this biosensor was saturated slower by glucose, so in logarithmic coordinates linear range of glucose determination was from 1 mM to 9 mM. Generally, these biosensors' behavior (especially for the ATP determination) corresponds with the results obtained with drop-casting of polymer mixture without electrospinning in the previous part of the present work – in both cases addition of GNPs led to higher sensitivity of the biosensors to ATP. These results also demonstrate that electrospinning of two-enzyme mixture is possible, and both enzymes retain their activities after being included into nanofibers. Although we did not measure directly enzyme activities after immobilizations, similar sensitivity of the nanofiber-based biosensors and

drop-casting-based biosensors to glucose and ATP means that activities of enzymes after immobilization in both cases were quite close.

Selectivity of glucose oxidase/hexokinase biosensors towards potential interfering molecules was tested earlier (Soldatkin et al. 2009). It was shown that both enzymes are highly selective and not sensitive to glucose or ATP analogues, such as monosaccharides, ADP, AMP, GTP and UTP.

It should be noted that the biosensor cannot simultaneously detect concentrations of both glucose and ATP. Concentration of one of the substances should be very low (<10  $\mu\text{M}$ ) or already known. This limitation is applicable for all biosensors based on GOx and HEX.



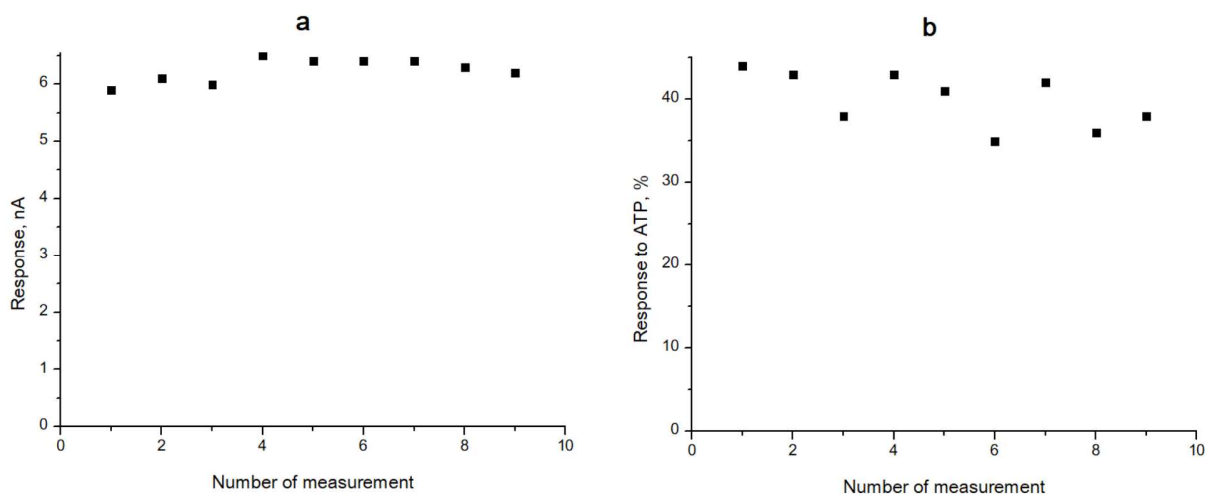
**Fig. 6** Calibration curves for the determination of glucose (a) and ATP (b) of the biosensors based on GOx and HEX entrapped into electrospun nanofibers with different concentrations of GNPs. Glucose determination was performed in the absence of ATP. ATP determination was performed in the presence of 100  $\mu\text{M}$  glucose

#### *Reproducibility and stability*

Reproducibility and stability of the biosensor responses are important parameters, which demonstrate the accuracy of the biosensor and the possibility of multiple measurements with the same biosensor, which makes biosensor analysis much cheaper. In order to evaluate those parameters, we prepared biosensors with the best composition (0.5% GOx, 2.5% HEX, 6.67% PVA-SbQ,  $5.5 \times 10^8$  GNPs/mL) and obtained 9 responses to glucose and ATP (Fig. 7). After each response, working buffer in the cell was changed 3 times to wash out substrates and products from the biosensor. Time of each response with washing was about 15 min, and all experiment took about 2.5 hours (although the responses and washing can be performed 2-3 times faster if needed, we made quite slow measurements since wanted to test the biosensor stability). Relative standard deviation of 9 responses to glucose was 3.4%, which demonstrate

that biosensor analysis is accurate and reproducible. Moreover, this experiment indirectly demonstrates that stability of the nanofibers on the electrode surface was good enough, because in the opposite case the biosensor responses would decrease dramatically.

On the other hand, RSD of responses to ATP was worse (8.3%) because the response is generated in a more complex way – by two simultaneous and competing enzymatic reactions. The responses to glucose were stable and did not change during measurements. But responses to ATP decreased slowly during the measurements – the final response was 14% lower than the initial one. This means that immobilized HEX is not stable and HEX either loses its activity or is washed out into the working buffer. Notably, molecular mass and, consequently, size of GOx (160 kDa) is larger than those for HEX (54 kDa for monomer form and 110 kDa for dimer form), and HEX can leak through the polymer network more easily. For this reason, an increased duration of UV irradiation can be used to create denser PVA-SbQ structure and prevent HEX leakage.



**Fig. 7** Reproducibility of the biosensor responses to glucose (a) and ATP (b). Response to glucose (100  $\mu$ M) was recorded in the absence of ATP. Response to ATP (100  $\mu$ M) was recorded in the presence of 100  $\mu$ M glucose

Storage of the biosensors was examined during 32 days. The biosensors were stored in a dry frozen state at  $-18^{\circ}\text{C}$ . After storage, responses to glucose did not change, while responses to ATP decreased to 51% of initial value. So big difference is caused by the fact that GOx is quite stable enzyme, while HEX has much worse stability. Such difference between storage of GOx and HEX was also demonstrated in case of GOx/HEX cross-linking by glutaraldehyde (Kucherenko et al. 2014).

## Conclusions

In the present study, a simple and rapid method for co-immobilization of two enzymes (GOx and HEX) during electrospinning of PVA-SbQ/GNP mixture was proposed. After electrospinning and UV irradiation, both enzymes were entrapped in the nanofibers. Optimal duration of electrospinning procedure for one biosensor was 5 min, time of UV irradiation was 10 min, thus total time of the biosensor preparation was about 15 min. Productivity of the proposed method was quite high – 100 µL of the enzyme/polymer solution was enough for preparation approximately 70-100 biosensors. The obtained biosensors were fully operational and their characteristics were not inferior to the characteristics of the similar biosensors prepared by drop-casting, which indicates good retention of the enzyme activity after the electrospinning procedure. Optimal composition of the mixture for electrospinning was 0.5% GOx, 2.5% HEX, 6.67% PVA-SbQ and  $5.5 \times 10^8$  GNPs/mL. Linear range of the glucose determination was from 10 µM to 3 mM, while that for the ATP was 25-200 µM. Relative standard deviation of the biosensors responses to glucose was 3.4% and to ATP was 8.3%. The biosensor potentially can be used for the detection of ATP in medications and can be applied in biological research, such as neurochemical experiments which need ATP sensing. Furthermore, it can be used for glucose detection in various media that do not contain significant concentrations of ATP (i.e., food products), even though this is not the primary purpose of the biosensor. The main advantage of the enzyme immobilization in electrospun nanofibers comparing to commonly utilized GA cross-linking is ability to quickly produce large number of biosensors. Furthermore, toxic compounds (i.e., GA) are not used during the biosensor preparation. The described electrospinning method can be easily modified by replacing GOx and HEX with other enzymes, which opens possibilities to one-step generation of enzyme-containing nanofibers for different purposes.

### **Acknowledgments**

The authors would like to thank INL institute (Institut de nanotechnologies de Lyon, France) for providing the facilities for the electrospinning technique, the required access to the electron microscopes facilities and for their remarkable assistance whenever it was needed.

### **Statements and declarations**

**Funding** This work was supported by the National Research Fund of Ukraine in the framework of the competition of projects for research and development "Support of research of leading and young scientists" (project 2020.02/0097) and NAS of Ukraine in the framework of target research program of NAS of Ukraine "Smart" sensor devices of the new generation based on modern materials and technologies". I. Kucherenko would like to acknowledge the French

Ministry of Higher Education and Research and Campus France for his PhD grant (Eiffel Scholarship).

**Conflict of interest** Authors declare that there is no conflict related to the financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

## References

- Bhardwaj N, Kundu SC (2010) Electrospinning: A fascinating fiber fabrication technique. *Biotechnology Advances* 28:325–347. <https://doi.org/10.1016/j.biotechadv.2010.01.004>
- Braiek M, Djebbi MA, Chateaux J-F, et al (2018) A conductometric creatinine biosensor prepared through contact printing of polyvinyl alcohol/polyethyleneimine based enzymatic membrane. *Microelectronic Engineering* 187–188:43–49. <https://doi.org/10.1016/j.mee.2017.11.018>
- Cai S, Chen X, Chen H, et al (2023) A fluorescent aptasensor for ATP based on functional DNAzyme/walker and terminal deoxynucleotidyl transferase-assisted formation of DNA-AgNCs. *Analyst* 148:799–805. <https://doi.org/10.1039/d2an02006h>
- Chigome S, Torto N (2011) A review of opportunities for electrospun nanofibers in analytical chemistry. *Analytica Chimica Acta* 706:25–36. <https://doi.org/10.1016/j.aca.2011.08.021>
- Compagnone D, Guilbault GG (1997) Glucose oxidase/hexokinase electrode for the determination of ATP. *Analytica Chimica Acta* 340:109–113. [https://doi.org/10.1016/S0003-2670\(96\)00451-5](https://doi.org/10.1016/S0003-2670(96)00451-5)
- Das P, Das M, Chinnadayala SR, et al (2016) Recent advances on developing 3rd generation enzyme electrode for biosensor applications. *Biosensors and Bioelectronics* 79:386–397. <https://doi.org/10.1016/j.bios.2015.12.055>
- Demirci Uzun S, Kayaci F, Uyar T, et al (2014) Bioactive Surface Design Based on Functional Composite Electrospun Nanofibers for Biomolecule Immobilization and Biosensor Applications. *ACS Applied Materials & Interfaces* 6:5235–5243. <https://doi.org/10.1021/am5005927>
- Dridi F, Marrakchi M, Gargouri M, et al (2015) Thermolysin entrapped in a gold nanoparticles/polymer composite for direct and sensitive conductometric biosensing of ochratoxin A in olive oil. *Sensors and Actuators B: Chemical* 221:480–490. <https://doi.org/10.1016/j.snb.2015.06.120>
- El-Moghazy AY, Soliman EA, Ibrahim HZ, et al (2016) Biosensor based on electrospun blended chitosan-poly (vinyl alcohol) nanofibrous enzymatically sensitized membranes for pirimiphos-methyl detection in olive oil. *Talanta* 155:258–264. <https://doi.org/10.1016/j.talanta.2016.04.018>
- Guo F, Xu XX, Sun ZZ, et al (2011) A novel amperometric hydrogen peroxide biosensor based on electrospun Hb–collagen composite. *Colloids and Surfaces B: Biointerfaces* 86:140–145. <https://doi.org/10.1016/j.colsurfb.2011.03.032>
- Jiang P, Wang Y, Zhao L, et al (2018) Applications of Gold Nanoparticles in Non-Optical Biosensors. *Nanomaterials (Basel)* 8:977. <https://doi.org/10.3390/nano8120977>



- Jouha J, Li F, Xiong H (2023) A fluorescence biosensor based on DNA aptamers-COF for highly selective detection of ATP and thrombin. *Spectrochim Acta A Mol Biomol Spectrosc* 295:122615. <https://doi.org/10.1016/j.saa.2023.122615>
- Kirsch J, Siltanen C, Zhou Q, et al (2013) Biosensor technology: recent advances in threat agent detection and medicine. *Chemical Society Reviews* 42:8733. <https://doi.org/10.1039/c3cs60141b>
- Kucherenko IS, Didukh DY, Soldatkin OO, Soldatkin AP (2014) Amperometric biosensor system for simultaneous determination of adenosine-5'-triphosphate and glucose. *Analytical Chemistry* 86:. <https://doi.org/10.1021/ac5006553>
- Kucherenko IS, Farre C, Raimondi G, et al (2017) Gold Nanoparticle/Polymer/Enzyme Nanocomposite for the Development of Adenosine Triphosphate Biosensor. In: Fesenko O, Yatsenko L (eds) *Nanophysics, Nanomaterials, Interface Studies, and Applications*. Springer International Publishing, Cham, pp 533–545
- Li D, Pang Z, Chen X, et al (2014) A catechol biosensor based on electrospun carbon nanofibers. *Beilstein Journal of Nanotechnology* 5:346–354. <https://doi.org/10.3762/bjnano.5.39>
- Lombardi M, Gabrielli M, Adinolfi E, Verderio C (2021) Role of ATP in Extracellular Vesicle Biogenesis and Dynamics. *Frontiers in Pharmacology* 12:
- Lu Y, Li J, Liu Y, et al (2023) Bi-enzyme competition based on ZIF-67 co-immobilization for real-time monitoring of exocellular ATP. *Mikrochim Acta* 190:71. <https://doi.org/10.1007/s00604-023-05652-y>
- Manesh KM, Kim HT, Santhosh P, et al (2008) A novel glucose biosensor based on immobilization of glucose oxidase into multiwall carbon nanotubes–polyelectrolyte-loaded electrospun nanofibrous membrane. *Biosensors and Bioelectronics* 23:771–779. <https://doi.org/10.1016/j.bios.2007.08.016>
- Marty JL, Mionetto N, Rouillon R (1992) Entrapped Enzymes in Photocrosslinkable gel for Enzyme Electrodes. *Analytical Letters* 25:1389–1398. <https://doi.org/10.1080/00032719208017123>
- Marx S, Jose M V., Andersen JD, Russell AJ (2011) Electrospun gold nanofiber electrodes for biosensors. *Biosensors and Bioelectronics* 26:2981–2986. <https://doi.org/10.1016/j.bios.2010.11.050>
- Moreno-Cortez IE, Romero-García J, González-González V, et al (2015) Encapsulation and immobilization of papain in electrospun nanofibrous membranes of PVA cross-linked with glutaraldehyde vapor. *Materials Science and Engineering: C* 52:306–314. <https://doi.org/10.1016/j.msec.2015.03.049>
- Numnuam A, Thavarungkul P, Kanatharana P (2014) An amperometric uric acid biosensor based on chitosan-carbon nanotubes electrospun nanofiber on silver nanoparticles. *Analytical and Bioanalytical Chemistry* 406:3763–3772. <https://doi.org/10.1007/s00216-014-7770-3>
- Pinna S, Kunz C, Halpern A, et al (2022) A prebiotic basis for ATP as the universal energy currency. *PLOS Biology* 20:e3001437. <https://doi.org/10.1371/journal.pbio.3001437>

- Pyeshkova VM, Dudchenko OY, Soldatkin OO, et al (2022) Development of three-enzyme lactose amperometric biosensor modified by nanosized poly (meta-phenylenediamine) film. *Appl Nanosci* 12:1267–1274. <https://doi.org/10.1007/s13204-021-01859-8>
- Ren G, Xu X, Liu Q, et al (2006) Electrospun poly(vinyl alcohol)/glucose oxidase biocomposite membranes for biosensor applications. *Reactive and Functional Polymers* 66:1559–1564. <https://doi.org/10.1016/j.reactfunctpolym.2006.05.005>
- Ronkainen NJ, Halsall HB, Heineman WR (2010) Electrochemical biosensors. *Chemical Society Reviews* 39:1747. <https://doi.org/10.1039/b714449k>
- RoyChoudhury S, Umasankar Y, Hutcheson JD, et al (2018) Uricase Based Enzymatic Biosensor for Non-invasive Detection of Uric Acid by Entrapment in PVA-SbQ Polymer Matrix. *Electroanalysis* 30:2374–2385. <https://doi.org/10.1002/elan.201800360>
- Sapountzi E, Braiek M, Farre C, et al (2015) One-Step Fabrication of Electrospun Photo-Cross-Linkable Polymer Nanofibers Incorporating Multiwall Carbon Nanotubes and Enzyme for Biosensing. *Journal of The Electrochemical Society* 162:B275–B281. <https://doi.org/10.1149/2.0831510jes>
- Sapountzi E, Braiek M, Vocanson F, et al (2017) Gold nanoparticles assembly on electrospun poly(vinyl alcohol)/poly(ethyleneimine)/glucose oxidase nanofibers for ultrasensitive electrochemical glucose biosensing. *Sensors and Actuators B: Chemical* 238:392–401. <https://doi.org/10.1016/j.snb.2016.07.062>
- Sassolas A, Blum LJ, Leca-Bouvier BD (2012) Immobilization strategies to develop enzymatic biosensors. *Biotechnology Advances* 30:489–511. <https://doi.org/10.1016/j.biotechadv.2011.09.003>
- Shin YJ, Kameoka J (2012) Amperometric cholesterol biosensor using layer-by-layer adsorption technique onto electrospun polyaniline nanofibers. *Journal of Industrial and Engineering Chemistry* 18:193–197. <https://doi.org/10.1016/j.jiec.2011.11.009>
- Si Q, Li Y, Huang Z, et al (2023) Construction of a simple dual-mode ATP-sensing system for reliable fish freshness evaluation. *Anal Chim Acta* 1252:341048. <https://doi.org/10.1016/j.aca.2023.341048>
- Soldatkin OO, Schuvailo OM, Marinesco S, et al (2009) Microbiosensor based on glucose oxidase and hexokinase co-immobilised on platinum microelectrode for selective ATP detection. *Talanta* 78:1023–1028. <https://doi.org/10.1016/j.talanta.2009.01.008>
- Soldatkina OV, Kucherenko IS, Pyeshkova VM, et al (2017) Improvement of amperometric transducer selectivity using nanosized phenylenediamine films. *Nanoscale Res Lett* 12:594. <https://doi.org/10.1186/s11671-017-2353-9>
- Soldatkina OV, Kucherenko IS, Soldatkin OO, et al (2019) Development of electrochemical biosensors with various types of zeolites. *Appl Nanosci* 9:737–747. <https://doi.org/10.1007/s13204-018-0725-9>
- Su X, Wei J, Ren X, et al (2013) A New Amperometric Glucose Biosensor Based on One-Step Electrospun Poly(Vinyl Alcohol)/Chitosan Nanofibers. *Journal of Biomedical Nanotechnology* 9:1776–1783. <https://doi.org/10.1166/jbn.2013.1671>

- Thenmozhi S, Dharmaraj N, Kadirvelu K, Kim HY (2017) Electrospun nanofibers: New generation materials for advanced applications. *Materials Science and Engineering: B* 217:36–48. <https://doi.org/10.1016/j.mseb.2017.01.001>
- Wang Z-G, Wan L-S, Liu Z-M, et al (2009) Enzyme immobilization on electrospun polymer nanofibers: An overview. *Journal of Molecular Catalysis B: Enzymatic* 56:189–195. <https://doi.org/10.1016/j.molcatb.2008.05.005>
- Zhao L, Liu Q, Yan S, et al (2013) Multimeric immobilization of alcohol oxidase on electrospun fibers for valid tests of alcoholic saliva. *Journal of Biotechnology* 168:46–54. <https://doi.org/10.1016/j.jbiotec.2013.08.015>
- Zhu Q, Liang B, Liang Y, et al (2020) 3D bimetallic Au/Pt nanoflowers decorated needle-type microelectrode for direct in situ monitoring of ATP secreted from living cells. *Biosens Bioelectron* 153:112019. <https://doi.org/10.1016/j.bios.2020.112019>