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Comparison of Three Methods of Biocompatible Multi-Walled Carbon Nanotubes Confinement for the Development of Implantable Amperometric Adenosine-5′-Triphosphate Biosensors

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An amperometric biosensor for adenosine 5′-triphosphate (ATP) was developed applying a competitive assay of two glucose converting enzymes: glucose oxidase (GOD) and hexokinase (HEX). Competition between GOD and HEX for glucose in presence of ATP, lead to a decrease in the current coming from the hydrogen peroxide generated by the GOD, and allows ATP detection. The biosensor was realized on commercial screen-printed electrodes modified with carbon nanotubes (CNTs). Confinement of CNTs in a polycarbonate membrane, Chitosan and Nafion polymers was investigated as possible solutions for implantable sensors. Nafion gave the best performances, with a sensitivity of 25 pA/μM mm^{-2}, and a detection limit of 257 μM. The sensor resulted able to measure ATP concentrations in the range of hundred of μmol/l.

Keywords:

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1. INTRODUCTION

Adenosine-5′-triphosphate (ATP) is a multifunctional nucleotide used by cells as an energy source for many important reactions. In addition, extracellular ATP may affect many biological processes like neurotransmission, muscle contraction, vascular tone and immunomodulation;[1] moreover, extracellular ATP plays a critical role in the physiological regulation of inflammation and in the protection of tissues from excessive damage.[2,3]

Extracellular concentration of ATP may vary a lot according to the nature of tissue damage: from few nmol/l, in case of simple inflammation,[4] to several μmol/l in case of developing tumors.[5]

For this reason, monitoring the extracellular ATP can be a good strategy to know the status of inflammation of a tissue, and personalize the therapy to yield the maximum efficacy.

ATP is usually sensed with spectrophotometry,[6] liquid chromatography,[7] fluorescence,[8] chemiluminescence,[9] bioluminescence[10] and with amperometric biosensors.[11]

Electrochemical detection of ATP has a wide range of applicability in physiological studies, especially in those directed towards *in vivo* applications, due to the possibility to integrate the sensor and the inexpensive cost of its realization with volume production.

The main strategy employed today for the electrochemical detection of ATP is based on a combination of an ATP-dependent enzyme with an ATP-independent enzyme. The principal approaches for the 2-enzyme systems are based on glycerol kinase/glycerol oxidase[12] and on glucose oxidase/hexokinase.[13,14] Other ways of ATP detection are based on the employ of the H^+-ATPase,[15] choline kinase[16] and Apyrase.[17]

Carbon nanotubes (CNT) have been shown to be instrumental in biosensor applications, thanks to their property to enhance the output current. It has been shown, that nanotubes greatly increase the sensor performance, improving sensitivity and detection limit.[18,19] However, one drawback of this approach is the toxicity of CNTs *in vivo*: the nanofibers accumulate quickly in the organs generating inflammatory responses.[20] Moreover, the novelty of this material makes the estimation of the long-term effects of CNT accumulation in biological tissues still

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not possible. For these reasons, CNTs cannot be used for *in vivo* sensors without reliable means to prevent their dispersion in the body. A possible solution to this problem is the confinement of carbon nanotubes with a polymeric matrix.

In this work we compare three different ways of confining CNTs:

(1) coverage of drop cast CNTs with a commercial polycarbonate membrane;
(2) drop cast of CNTs entrapped in a Nafion matrix and
(3) drop cast of CNTs entrapped in a Chitosan matrix.

The employment of the polycarbonate membrane is a common approach used in the development of implantable biosensors;[21] Chitosan is a natural polysaccharide with unique biological properties including non-toxicity, physiological inertness, affinity to proteins, hemostatic fungistatic and antitumoral properties,[22] while Nafion is an artificial polymer characterized by biocompatibility, excellent ion exchange properties and permselectivity.[23]

The three methods have been used to realize an ATP biosensor based on *glucose oxidase* (GOD) and *hexokinase* (HEX). Enzymes compete for the substrate glucose, enabling quantitative estimation of ATP. In presence of glucose, GOD generates hydrogen peroxide (H_2O_2), which is oxidized at the electrode surface; in presence of ATP, the hexokinase produce glucose-6-phosphate, a compound not detected by the electrode, which leads to a decrease of the H_2O_2 current signal. The glucose oxidase/hexokinase strategy has been chosen because of the high availability of glucose in all the biological samples.

2. MATERIAL AND METHODS

2.1. Reagents

Gold screen printed electrodes (model DRP 250AT) and multiwalled carbon nanotubes powder (MWCNT-diameter 10 nm, lengths 1–2 uM, COOH content 5%), were purchased from Dropsens. Glucose oxidase from *Aspergillus Niger*; hexokinase type 3 from *Baker Yeast*, D-(+) glucose and Chitosan (low molecular weight) were obtained from Sigma. ATP and Nafion were bought form Aldrich. Polycarbonate membrane, diameter 13 mm, pore size 0.1 μm was bought from GE water and process technologies.

2.2. Solution Preparation

GOD and HEX were diluted to the stock concentration of 30 mg/ml in PBS buffer pH 7.4.

Glucose was diluted in PBS to the concentration of 1 M and kept at 4 °C overnight before the first use, in order to allow the mutarotation of the molecules in solution.

Chitosan was diluted in a 2% water solution acetic acid to the concentration of 0.5% (w/v) and kept at 4 °C; Chitosan-CNT solution was obtained diluting the CNT at the concentration of 10 mg/ml, and sonicating until homogeneous dispersion of the macro aggregates.

Nafion was diluted in a 50% water, 50% ethanol solution to the concentration of 0.5% (w/v); Nafion-CNT solution have been prepared diluting the CNT in the Nafion solution at the concentration of 10 mg/ml and sonicated for 20 minutes. Chloroform CNT were prepared diluting the nanotubes in chloroform at the concentration of 1 mg/ml and then sonicated for 20 minutes in order to break the macro aggregates.

ATP was diluted to the concentration of 50 mM in PBS pH 5.8 and kept at −20 °C when not used.

2.3. Electrode Preparation

The electrodes were made of a gold working electrode, a platinum counter electrode and a silver reference electrode. The working electrode area was 12.56 mm^2, while the total area of the cell, 22 mm^2. CNT nanostructuring was obtained by drop cast of 30 μg of CNT solutions on the working electrode until complete evaporation of the solvent. Glucose oxidase and hexokinase were then mixed in a 1:1 ratio to obtain a solution with 15 mg/ml of each protein. 20 μl of the solution were then drop cast onto the working electrode, and let dry at 4 °C overnight.

Polycarbonate membrane was previously dipped in water for 5 minutes and stuck to the electrode with capillary force; immobilization was done gluing the edge of the membrane outside the electrode with silicone.

2.4. Electrochemical Measurement

The electrochemical response of the electrodes was investigated by chronoamperometry under aerobic conditions and in presence of mild solution stirring (100 rpm). Chronoamperometries were acquired at the potential of + 650 mV versus Ag/AgCl, using a Versastat 3 Potentiostat (Princeton Applied Technologies). The electrode was dipped in 25 ml of PBS 1×, MgCl 5 mM pH 7.4. Mg^{2+} ions are necessary for the catalysis mediated by the hexokinase. The optimum concentration of the ion was previously described by Compagnone and Guilbault.[24]

Glucose and ATP samples were then injected in the solution during the chronoamperometry.

3. RESULTS AND DISCUSSION

3.1. Sensing Principle

The bio-element of our biosensor consists of two co-immobilized enzymes: glucose oxidase and hexokinase. Both enzymes are sensitive to glucose, but with a different catalytic mechanism:

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2$$

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HEX}} \text{D-glucose-6-P} + \text{ADP}$$

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The current detected is generated by the discharge of the hydrogen peroxide at the electrode surface, according to the following mechanism:

$$H_2O_2 \xrightarrow{+650\ mV} 2H^+ + O_2 + 2e^-$$

If only glucose is injected, the electrochemical response of the biosensor becomes proportional to the glucose concentration in the media; in presence of ATP, the hexokinase competes with the glucose oxidase for the substrate, the quantity of hydrogen peroxide decreases and the electrochemical signal is reduced. We initially measured the output current for glucose. Once the current was stabilized, we recorded the current variation in presence of increasing concentrations of ATP, from 200 μM to 1 mM. The behavior of the current during the chronoamperometry is shown in Figure 1: after each ATP injection the signal decreased slightly to a new steady state value.

3.2. Effect of Carbon Nanotubes Addition on Biosensor Performance

CNT deposition on the electrode (CNT nanostructuration) resulted to be useful for biosensor developement, thanks to their property to enhance the output current. Before considering different methods of CNT confinement, we wanted to compare the performance of an ATP biosensor prepared without CNT, with one prepared with drop cast of carbon nanotubes dispersed in chloroform, a technique we successfully used to develop biosensors based on P450.[25] The current response of the biosensors for different ATP concentration is shown in Figure 2.

The addition of carbon nanotubes resulted in a 4× increase in sensitivity.

3.3. Effect of Enzymes Coimmobilization

Figure 3 shows the chronoamperometries obtained at +650 mV with a biosensor prepared with 15 mg/ml of glucose oxidase (gray line) and 15 mg/ml glucose oxidase + 15 mg/ml hexokinase (black line and inset), and CNT entrapped in chitosan. The sensors were tested with 2 injections of 12.5 μl of glucose 1 M. When the hexokinase is added, the output current for the glucose response is reduced to 2.7%, suggesting that the presence of the second protein is lowering the biosensor performance. This decrease in current was noted also when the total protein concentration on the top of electrode was maintained unvaried between the first and the second sensor, excluding the possibility of an excessive protein amount. We conclude that hexokinase have a strong disturbing effect on the performance of the biosensor. A possible explanation is that the protein adsorption onto the electrode surface could have created protein interactions that can limit the enzyme efficiency.

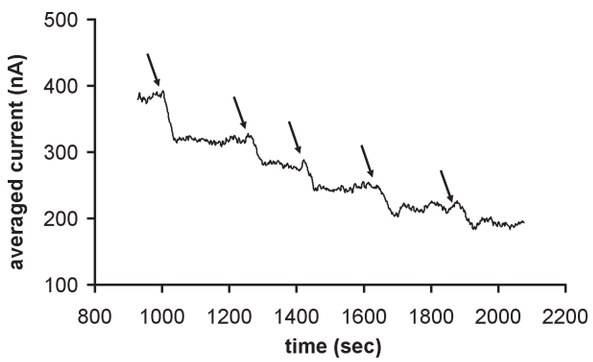

Fig. 1. Current response following analyte injections. Arrows mark the injection time. First injection: 12.5 μl of glucose 1 M; others, 200 μl of ATP 50 mM. Raw data was filtered applying a moving average transformation: each point in the graph is the average of 35 adjacent points in the raw data set.

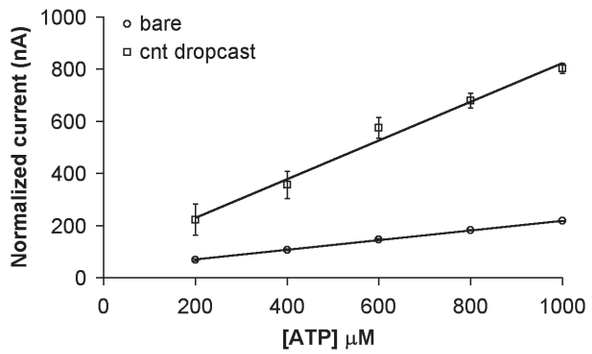

Fig. 2. Current response in ATP biosensors with and without CNT. Squares: GOD/HEX biosensor with nanostructuration of CNT dispersed in a chloroform solution; circles, ATP biosensor prepared on a bare electrode. Error bars represent instrument noise.

3.4. Biosensor Response with Phisiological Glucose Concentrations

In order to test the ATP biosensor in closer physiological conditions with animal models, the measurements were repeated in solutions with glucose concentrations ranging

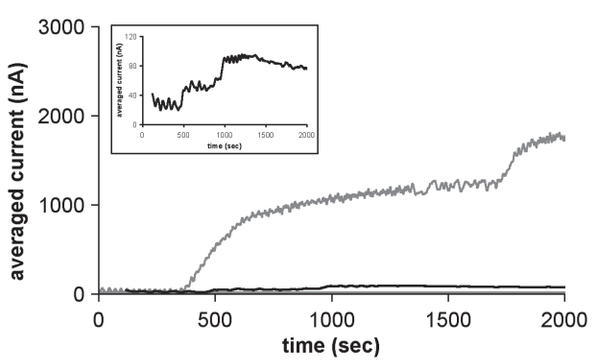

Fig. 3. Comparison of the glucose current response between single and double enzyme immobilization. Gray line: GOD sensor; Black line and inset, GOD + HEX sensor.

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from 1 to 3 mM, which represent values typically found in rat interstitium.[26]

Figure 4 shows the ATP response for a single biosensor tested at different glucose concentrations.

For each response, we calculated the sensitivity according to the Eq. (1)

$$S = \frac{1}{A}\frac{d(i_{ss} - i_{gl})}{dC} \quad (1)$$

Where S is the sensitivity, A is the working electrode area, i_{ss} is the steady state current reached after each ATP injection, i_{gl} is the steady state current reached after the glucose injection and C is the ATP concentration.

We obtained a sensitivity of 26.8 pA/μA mm^{-2} for glucose 1 mM, 23.9 pA/μA mm^{-2} for glucose 2 mM, and 12.5 pA/μA mm^{-2} for glucose 3 mM. The halving of the ATP sensitivity with glucose 3 mM may be caused by the saturation of the sensor to the substrate: for the same ATP concentrations, an increased availability of glucose is reducing the current decrease operated by hexokinase, leading to an absence of the response/concentration proportionality. A similar effect was already observed with the GOD/HEX sensor developed by Soldatkin et al.[11] This behavior was also confirmed in another experiment in which we repeated twice the glucose/ATP injection cycle during the same measurement (Fig. 5). During the second injection, the biosensor becomes insensitive to glucose, as we did not record a relevant increase in the current. Also, sensitivity versus ATP varied significantly between the first and the second injection cycle: from 23.1 to 12.9 pA/μM mm^2.

Obtained sensitivities are comparable with the values shown before. Nevertheless, we cannot exclude that in this case also the total ATP concentration could have affected the biosensor response: high ATP levels could have saturated the Hexokinase, leading to a decrease in sensitivity.

Given the differences of response according to the glucose concentration, we conclude that in order to get a reliable estimation, it is necessary to constantly monitor the glucose level in an independent manner, for example coupling a second glucose sensor which constantly follows

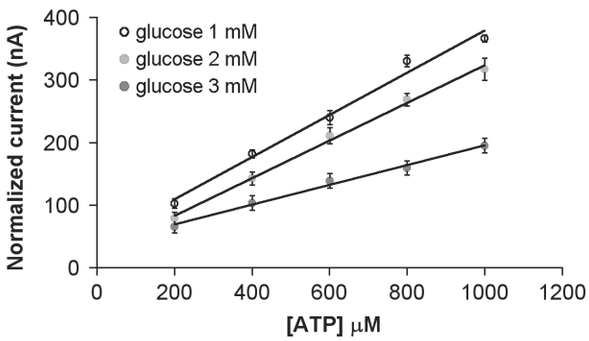

Fig. 4. Calibration curves for the ATP response from 0 to 1000 μM at different glucose concentrations. Error bars represent instrument noise.

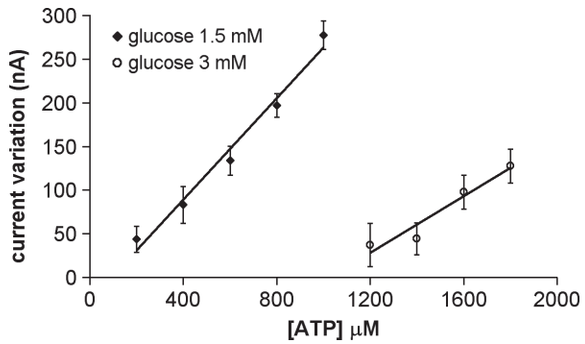

Fig. 5. Effect of two different glucose concentrations on the ATP detection during the same experiment.

the glucose oscillations. Knowing if the baseline current is stable with an independent sensor would help us to estimate more precisely if the current variation is due to a decrease of glucose or to an increase of ATP when the concentrations in the sample are not known *a priori*.

An additional advantage in the employment of an independent sensor to monitor the baseline current is to make the ATP biosensor not subjected to the interference of the molecules oxidized at + 650 mV versus Ag/AgCl, like catechols or ascorbic acid: in the GOD/HEX biosensor, the ATP detection is done on a subtractive basis, detecting a current decrease due to the hydrogen peroxide deprivation caused by the activation of the hexokinase. Contaminants oxidized at + 650 mV will only affect the initial baseline current which is monitored with another sensor.

3.5. Effect of Different CNT Nanostructurations on the Biosensor Response

The deposition of CNT on the electrode (CNT nanostructuration), represent a major issue for the development of implantable biosensors, since accidental release of nanotubes in biological tissues leads to inflammatory response. We compared three different methods of CNT confinement: coverage with a polycarbonate membrane; entrapment in Chitosan, and entrapment in Nafion, with a simple drop cast of CNT in chloroform solution. For each sensor, we calculated the current variation for ATP, averaging four different measurements with the same electrode, to obtain sensitivity, detection limit and response time.

Sensitivity estimations were calculated according to the Eq. (1)

Detection limit was calculated using the IUPAC definition of Mc Naught and Wilkinson[27]

$$L.O.D. = \frac{k\delta i}{S} \quad (2)$$

Where $L.O.D.$ is the limit of detection, δi is the standard deviation for the current measurement, S is the detector sensitivity, and k is a parameter accounting for the confidence level ($k = 1, 2$, or 3 corresponds to 68.2%, 95.4%, or 99.6% of statistical confidence).

As response time, we considered the time necessary to reach the 90% of the steady state response after each analyte injection, according to the following Eq. (3):

$$\begin{cases} \Delta t = t_1 - t_0 \\ i(t_1) = i(t_0) - 0.9[i(t_0) - i(t \to \infty)] \end{cases} \quad (3)$$

Where Δt is the response time, t_0 time of the analyte injection and t_1 is the time in which the 90% of the steady state response is reached.

Table I resumes the average sensitivities and detection limit for all the methods considered.

All the proposed techniques have shown high noise during measurements, setting the detection limit at high concentrations. Chloroform CNT showed, as expected, the fastest response time to the analytes and the lowest variability in terms of reproducibility. We have recently shown[25] how the enzymes bind tightly to the nanotubes surface, forming a protein monolayer which minimizes the distance between the protein and the electrode. This configuration can reduce the time required to the hydrogen peroxide to diffuse at the electrode, and puts the enzymatic layer in direct contact with the solution, lowering the time necessary to the analyte to reach the enzymes.

The addition of a protective polycarbonate membrane didn't affect significantly the sensitivity or the detection limit. However, the response time to the analytes increased of ten fold for glucose and 5 times respect to the ATP. CNT entrapment in Chitosan and Nafion also affected the response time against ATP and glucose, which doubled in respect to the chloroform CNTs, but resulted 3 times faster compared to the confinement of CNTs in polycarbonate. Among the three methods of confinement, Nafion showed the fastest response to ATP, with 114.5 ± 55.7 seconds, and a better time reproducibility (see Table I). However, due to the large variability between the measurements, response times for the methods considered do not statistically differ. Nevertheless, a response time in the time-scale of hundred of seconds does not represent a critical issue to track concentration changes, since analyte variations are monitored in a longer time span.

In terms of detection limit and sensitivity, Chitosan gave the best results, with an average detection limit of 257.3 μM, a sensitivity of 25.3 pA/μM mm^2.

Table I. Sensitivity, detection limit and response time for the different CNT nanostructurations.

	Sensitivity pA/μM mm^{-2}	Detection limit $K = 1$ (μM)	Response time glucose (sec)	Response time ATP (sec)
Chloroform CNT	17.8 ± 3.05	467 ± 65.0	47 ± 17.9	58.5 ± 23.9
Polycarbonate	16.8 ± 2.42	518.5 ± 83.9	452 ± 91.9	258.7 ± 147.3
Nafion CNT	13.5 ± 6.54	358.7 ± 183.9	138 ± 72.1	114.5 ± 55.7
Chitosan CNT	25.3 ± 4.65	257.3 ± 72.6	129 ± 99.5	119.7 ± 84.3

In a separate experiment we compared the biosensor performance after several days.

For each nanostructuration we prepared a new electrode, and for each one we calculated the sensitivity after 1, 4 and 8 days after the preparation. When not measured, the electrodes were kept at 4 °C in a 1× PBS solution.

For each electrode we calculated the percentual variation respect the value obtained the day 1, which was fixed at 100%. Results are shown in Table II.

Polycarbonate membrane and Nafion become un-responsive after the day 4. Of the two, it was interesting to note that while Nafion showed a huge current decrease at the day 4, sensitivity doubled for the electrode with the polycarbonate membrane.

Nanostructuration with drop cast CNT presented a sensitivity decrease at the day 4 and a slight increase respect the first day at the day 8; Chitosan sensitivity decreased of one third at the day 4 and remained stable after four additional days.

Chitosan and Nafion have shown a behavior that can be easily explained: part of the nanostructuration could have been probably degraded during the days: Chitosan for example at in solutions with acidic and slightly basic pH tends to be protonated and therefore water soluble. However, in case of Chitosan, this effect seems to stabilize after day 4, since sensitivities do not change significantly after four additional days.

In conclusion, Chitosan showed the lowest detection limit and the highest sensitivity. Moreover, among the other confinement methods, Chitosan resulted to be the only one still active after 8 days. Current response drop of two thirds between day 1 and day 4, but didn't showed significant variations in the next days. These findings, together with the high biocompatibility and the high affinity to proteins of this material, led us to consider the entrapment of CNT and enzymes in Chitosan the best strategy for the development of an *in-vivo* sensor among the methods considered.

It has been shown that tumor interstitium has glucose concentrations in the range of few hundred of μM/l,[28] and accumulates ATP at high concentrations.[5] Our sensor, which resulted sensitive to micro-molar amounts of ATP, and operates with better efficiency with glucose concentrations below 1 mM, is therefore able to detect pathological concentrations of ATP, and ideally can find an application in the monitoring of ATP in tumors, as a tool to personalize the anticancer therapy. For example,

Table II. Performance of the different CNT nanostructurations in long term measurements.

	Day 1 (%)	Day 4 (%)	Day 8
Chloroform CNT	100	66.8	114.4%
Polycarbonate	100	228.6	Not responsive
Nafion CNT	100	14.2	Not responsive
Chitosan CNT	100	32.3	38.5

it is known that ATP hydrolysis favors the tumor growth causing release of the metalloprotease MMP9,[29] which facilitates tumor invasion, and expression of indoleamine oxygenase,[30] which has immunosuppressive activity. In the perspective of a therapy, which inhibits the ATP degradation, the sensor could be used to monitor the stability of ATP in the tumor microenvironment, and therefore validate the efficacy of the treatment.

4. CONCLUSIONS

In this work we have presented an ATP biosensor based on the glucose oxidase/hexokinase enzyme co-immobilization. Competition between GOD and HEX for glucose in presence of ATP, leads to a decrease in the current coming from the hydrogen peroxide generated by the GOD, allowing ATP detection. The sensor was realized onto commercial screen printed electrodes modified with carbon nanotubes. Sensitivity, detection limit and response time of 3 different methods of CNT confining were studied as possible solution for *in-vivo* applications of carbon nanotubes. Chitosan resulted to be the best among the materials considered to entrap CNT, thanks to the highest sensitivity, the lowest detection limit and its high biocompatibility and higher stability to long term measurements. Obtained response time at the considered stirring conditions for the various confinement methods, was in the order of hundred of seconds. However slow response times do not represent an issue for *in-vivo* monitoring, where variations of analytes are considered in a much larger time period, like hours or days. ATP response appeared to be dependent by the initial glucose concentration in the sample. Higher amounts of glucose decreased the ATP sensitivity.

The sensor was able to detect ATP concentrations of hundred of micromoles: although healthy tissues present extracellular ATP levels at very low concentrations,[11, 31] concentrations detectable with our sensor have been found the interstitium of solid tumors like the ovarian carcinoma.[5]

As putative application, our sensor can be used to monitor the ATP levels during therapeutic intervention.

Our group already realized a sensor based on P450 for the electrochemical detection of antitumor drugs.[25] P450-based drug detection and ATP monitoring could be integrated in a single array for personalized anti-tumor therapy.

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