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Long-term Biosensors for Metabolite Monitoring by using Carbon Nanotubes

***Cristina BOERO, Sandro CARRARA, Giovanni DE MICHELI**
Laboratory of Integrated Systems,
EPFL, 1015 Lausanne, Switzerland
*Tel.: (+41)-21-693-6878
E-mail: cristina.boero@epfl.ch

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Abstract: The key-point for the development of an amperometric sensor is the immobilization of the enzyme. In the present work we use biosensors based on *glucose oxidase* (GOD) onto electrodes nanostructured with *carbon nanotubes* (CNT), to be employed in cell culture monitoring. The goal is to determine the best immobilization strategy from the point-of-view of sensor lifetime. We compared three types of immobilization: the spontaneous adsorption of the enzyme on nanotubes, the entrapment in a Nafion matrix (optimizing also the quantity), and the cross-linking with glutaraldehyde. The cross-linking gives the best sensitivity, 17.38 μA mM^{-1} cm^{-2}, and the lowest detection limit, 25 μM. On the other hand, Nafion matrix allows to extend the linear range up to 7.5 mM. Finally, electrodes are tested over 35 days to analyze the lifetime. GOD cross-linking results to have 100% of retained activity after 35 days, while the adsorption and the entrapment retain only the 20 % of the original response.

Keywords: Electrochemical biosensors, Glucose, Carbon nanotubes, Immobilization, Nafion, Glutaraldehyde.

1. Introduction

Electrochemical enzyme-based biosensors have been largely used over the past few decades, because of their many advantages. First of all, they have a high specificity for the identification of a particular target molecule, due to enzyme selectivity. Then, they allow the direct transduction of a chemical reaction in an electrical measurement, which is easily detectable. These sensors have a high versatility, since the immobilization strategy used for one type of protein can be also employed for others of the

same family (i.e., oxidases). And finally, they can be easily miniaturized and used in disposable or even fully implantable devices.

Among the wide variety of amperometric sensors developed in the last 20 years, those for metabolite detection have had the best success. Glucose detection has attracted lot of researches [1], due to the importance that these instruments have in the life of diabetic patients. Other kinds of metabolites have also captured the interest, like lactate [2], related to anaerobic conditions due to hypoxia, cholesterol [3], important for the diagnosis and prevention of a number of clinical disorders, and glutamate [4], the major neurotransmitter in the brain.

Recently, an increased demand has arisen from the field of cell analysis. The monitoring of specific analytes involved in cell growth, cell proliferation and cell differentiation can contribute to a better understanding of the factors that influence metabolic processes, with a particular interest on stem cell mechanisms. In order to employ such biosensors to cell cultures, and in particular to stem cells, long-term stability plays a fundamental role, since they require a monitoring over several days. For example, mammalian cells can last for 5-10 days without contaminations [5]. Another interesting cell line is the one derived from the fusion of septal neurons with neuroblastoma cells. This cell line can mimic stem cell behavior, since it can be grown either in proliferation state, or it is possible to induce differentiation with some agents, like retinoic acid. These cells are able to survive up to 4 weeks [6].

Electrodes have been structured with different strategies, employing polymeric matrices, sol-gel, cross-linker, and mediators [7]. More recently, nanomaterials has been considered as alternative materials to wire the enzyme and the electrode surface, enhancing the electron transfer and the sensitivity of the sensors. Among the different nanomaterials, carbon nanotubes have revealed great electrical [8] and electrochemical [9] properties, suitable to be applied on biosensors. Many works have been published with the direct adsorption of the enzyme onto CNT surface [10]. Other researches have used polymer membranes to entrap the enzyme onto the electrode. Conductive polymers seem to be the best solution to develop amperometric biosensors, helping the exchange of charges. Nafion is a perfluorinated sulfonated cation exchanger, which has been widely used in electrochemical biosensors, due to its chemical inertness and its thermal stability, but especially for its anti-fouling properties [11]. Another immobilization strategy involves glutaraldehyde, an organic compound used as fixative, for its property to be a protein cross-linker.

All the aforementioned strategies has been combined together in order to develop more durable electrodes, with a long-term stability. To date, all commercially available glucometer have disposable electrode for glucose measurement. From the point-of-view of handling blood, the use of disposable sensors is an advantage [1], avoiding contamination problems and allowing safe measurements. On the other hand, long-term stability is an important issue for cell culture monitoring, as already mentioned. The sensor should be stable over the culture time, in order to accurately follow metabolite concentrations in different cell states. For this purpose, the stability of a sensor has to last at least 20 days.

The goal of the present work is a comparison between three different immobilization strategies of the enzyme for the development of a glucose biosensor with nanostructuration by using *Multi-Walled Carbon Nanotubes* (MWCNT). Glucose oxidase is let adsorbed onto the electrode surface, or entrapped in a Nafion matrix drop cast onto the electrode, or cross-linked with glutaraldehyde. Linear range, detection limit, sensitivity and lifetime are considered in order to evaluate the best immobilization strategy.

2. Experimental Section

2.1. Chemicals

Carbon paste screen-printed electrodes (SPE - model DRP-110) and multi-walled carbon nanotubes were purchased from Dropsens (Spain). The electrodes consists of a graphite working electrode, which presents an active area equal to about 13 mm^2, a counter electrode, also made of graphite, and a reference electrode, made of Ag/AgCl. The total area of the cell is 22 mm^2. Multi-walled carbon nanotubes (diameter 10 nm, length 1-2 μm) where purchased in powder (90 % purity), and subsequently diluted in chloroform to the concentration of 1 mg ml^{-1} [12]. Samples were then sonicated in order to obtain a homogeneous solution.

Glucose oxidase from *Aspergillus Niger* (GOD, EC 1.1.3.4, 129.9 units/mg solid), Nafion solution (5 wt% solution in a mixture of lower aliphatic alcohols and water), glutaraldehyde solution (25%), and D-(+)-glucose were purchased from Sigma-Aldrich (Switzerland). All the proteins were dissolved in *Phosphate Buffer Saline* (PBS) 0.01 M at pH 7.4, while glucose was dissolved in Milli-Q.

2.2. Preparation of Electrodes

Three types of electrodes were prepared in order to evaluate different strategies of protein immobilization. All the three were nanostructured by using MWCNT. For each electrode, 40 μl of the MWCNT-chloroform solution were deposited by drop casting (5 μl each time) onto the working electrode, and the electrodes were allowed to dry. Then, for the protein autonomously adsorbed onto the surface (CNT/GOD), 20 μl of glucose oxidase (15 mg ml^{-1}) were cast onto the working electrode and stored overnight at +4 ºC, in order to allow the adsorption of the protein onto the electrode surface. Then, the drop was rinsed out with Milli-Q. For the electrode with Nafion (CNT/GOD/Nafion), the procedure was similar to the previous one. Nafion was diluted at 0.5 wt% with a solution of 50 % of ethanol and 50 % of Milli-Q, according to what was reported in [13]. A drop of 2 μl of diluted Nafion was deposited onto the electrode and allowed to dry, forming a matrix of polymer which should protect the electrode from fouling and better immobilize the protein. For the immobilization with the cross-linker (CNT/GOD/Gluth), glutaraldehyde was diluted to 2.5 % with Milli-Q and the protein was dispersed in that solution (always with a concentration of 15 mg ml^{-1}) and a drop of 20 μl was deposited to cover the working electrode and stored overnight at +4 ºC. Then, the drop was rinsed out with Milli-Q. All the three electrodes were conditioned for 10 minutes at constant potential (+550 mV) before the first use and they were stored at +4 ºC, covered with PBS, when not used.

2.3 Apparatus

The electrochemical response of electrodes was investigated by chronoamperometries under aerobic conditions. Electrochemical measurements were acquired by using Versastat 3 potentiostat (Princeton Applied Technologies). For all the measurements, the electrode was dipped into a volume of 25 ml of PBS under stirring conditions. A volume of 25 μl per step of glucose was successively added into the solution with a time-step of 2 minutes. The applied potential was +550 mV vs Ag/AgCl.

3. Biosensor Characterization

3.1. Comparison for Different Immobilization Strategies

Calibration lines are derived from chronoamperometries within the concentration range from 0 to 4 mM of glucose. The substrate is dissolved in Milli-Q at a concentration of 0.5 M.

Chronoamperometries are carried out by using 25 ml of PBS as support electrolyte in stirring conditions. Screen-printed electrodes are dipped into the solution and 25 µl of the substrate are added, in order to obtain step of 0.5 mM. The addition are performed every 120 s, allowing the system to reach the steady-state. The response time of the system after each addition is around 30 s (data not shown). The three electrodes are tested in the same condition and calibration curves are shown in Fig. 1.

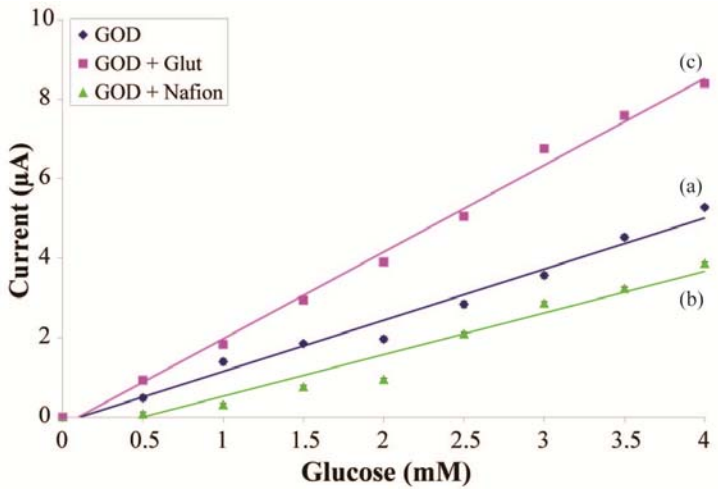

Fig. 1. Linear range: (a) adsorbed GOD; (b) GOD entrapped in Nafion; and (c) GOD cross-linked with glutaraldehyde. Data are represented as means ± Standard Deviation (SD).

The sensitivity related to GOD simply adsorbed onto MWCNT is 10.24 µA mM^{-1} cm^{-2}; the value related to GOD entrapped in Nafion is 8.18 µA mM^{-1} cm^{-2}; and finally, the sensitivity for GOD cross-linked with glutaraldehyde is 17.38 µA mM^{-1} cm^{-2}. Note from Fig. 1 that all the behaviors are almost linear within the concentration range. Fig. 2 illustrates the maximum linear range: the adsorption allows measurements from 100 µM to 6.5 mM, while the entrapment results in a linear range from 200 µM to 7.5 mM. The cross-linking shows the narrowest linear range, from 25 µM up to 5 mM, probably due to the fact that glutaraldehyde denaturates part of the enzymes, leaving a smaller amount able to react with the substrate. Regarding the case of Nafion, this fact is also confirmed by Rahman *et al.* [14]: they showed that electrodes modified with MWCNT and Nafion exhibit wider range compared to unmodified electrodes. On the other hand, the same electrodes show a higher inferior limit for the concentration window: the sensor seems to be insensitive for values lower than 200 µM, due to its anti-fouling properties. Nafion films were extensively used for their anti-interferent and anti-fouling properties in electrochemical sensing, since they work like an effective pre-selective barrier, eliminating anionic biological interference and enhancing the selectivity of the sensor. In particular, it has been used to avoid interferences due to ascorbic and uric acid [15]. Tsai *et al.* [11] showed that peak current decreases drastically if the glassy carbon electrode is coated with 0.5 wt% Nafion. It was because Nafion film becomes a barrier in diffusion on the surface of the electrode, as demonstrated by the detection limit. On the other hand, the presence of MWCNT improves significantly the sensitivity of the sensor, as demonstrated in our previous work [16].

The detection limit is also investigated to see what is the effect of different immobilization strategies, considering a signal-to-noise ratio of 3. Adsorbed GOD shows a limit of detection of 97 µM, entrapped enzyme results in a detection limit of 59 µM, and cross-linked oxidase has a detection limit of 16 µM. From a calibration point-of-view it seems that the cross-linking offers better advantages respect to the other techniques, even if the detection is not very linear for high concentration of glucose (refers to Fig. 2 curve c).

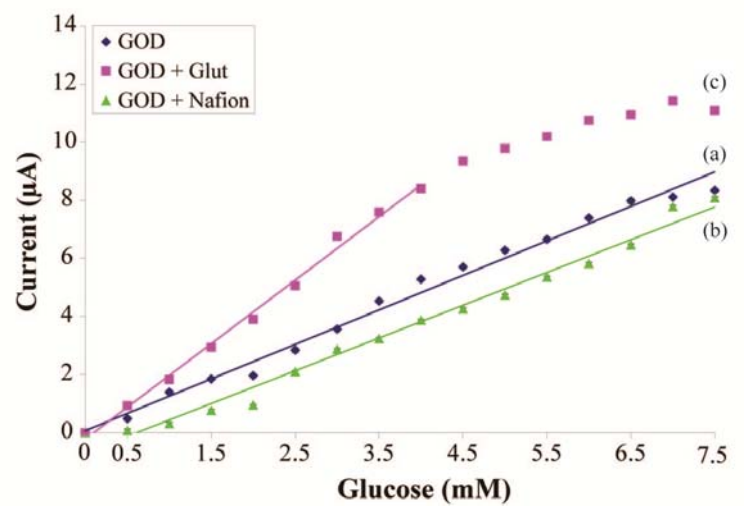

Fig. 2. Wider linear range: (a) adsorbed GOD; (b) GOD entrapped in Nafion; and (c) GOD cross-linked with glutaraldehyde. Data are represented as means ± SD.

3.2. Effect of Nafion Quantity on Electrocatalytic Properties of the Biosensor

We investigated the quantity of Nafion cast onto the electrode, in order to determine its influence on the biosensor response. Electrodes are prepared with carbon nanotubes, GOD and three different amounts of Nafion 0.5 wt% (volumes of 1, 2, and 3 µl). Fig. 3 illustrates calibration line for the developed biosensors.

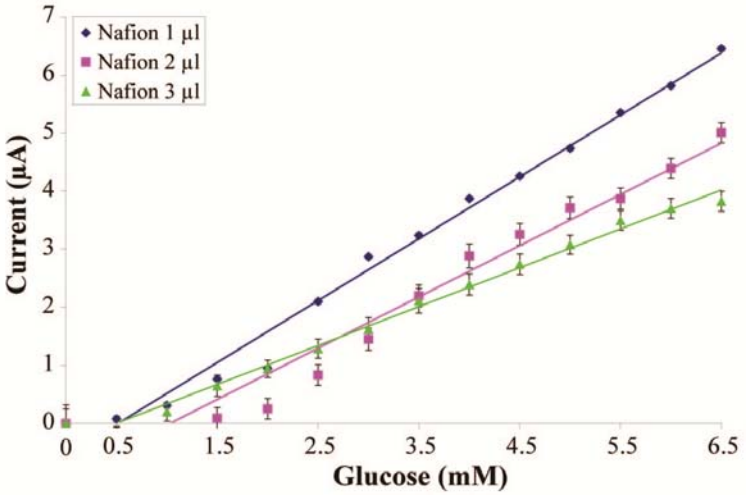

Fig. 3. Calibration line for GOD entrapped in different quantities of Nafion.
Data are represented as means ± SD.

It is possible to observe that sensitivity decreases with Nafion volume cast onto the surface, as shown also by Tang *et al.* [15]. The values of sensitivity for 1 µl, 2 µl, and 3 µl are 8.42 µA $mM^{-1}cm^{-2}$, 7.02 µA $mM^{-1}cm^{-2}$ and 5.33 µA $mM^{-1}cm^{-2}$, respectively. Note that sensitivity decreases drastically for higher volume of Nafion: the higher the volume, the thicker the matrix. Although Nafion is a cation exchanger, it obstructs glucose molecules to reach the electrode surface, and then glucose oxidase. Moreover, although the calculated limit of detection (S/N = 3) is around 60 µM for the three cases, the

lower limit of the linear range is around 1 mM. It seems to be not so dependent from the cast volume, but it confirms the tendency of Nafion to behave like a barrier in diffusion on the surface of the electrode, which is more evident for lower concentrations.

4. Sensors Lifetime

Measurements of glucose are carried out for 38 days, in order to verify the stability of the different strategies for enzyme immobilization. The three electrodes are tested for a range of concentration from 0.5 up to 4 mM every 2-3-4 days over 24 days. After a pause of 1 week, they are eventually tested to see the retained activity. Fig. 4, Fig. 5 and Fig. 6 illustrate the aging of the three immobilization strategies for 2.5 mM of glucose.

For the case of GOD cross-linking with glutaraldehyde, the 100 % of the retained activity is considered the one related to the 3rd day, because we observed a more evident increase of enzyme activity after some days respect to the other cases. Moreover, the measurement is unstable during the first ten days after the immobilization. It could be due to the fact that the cross-linking process is still under development, and the enzyme needs some days to stabilize.

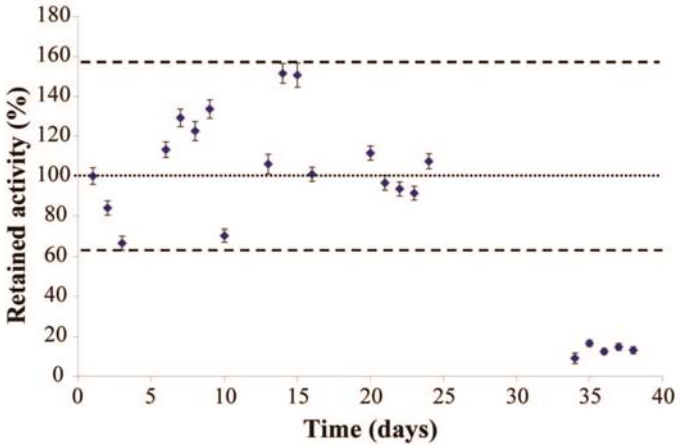

Fig. 4. Stability of the adsorbed GOD onto the electrode surface for the detection of 2.5 mM glucose. The applied potential was +550 mV. Data are represented as means ± SD.

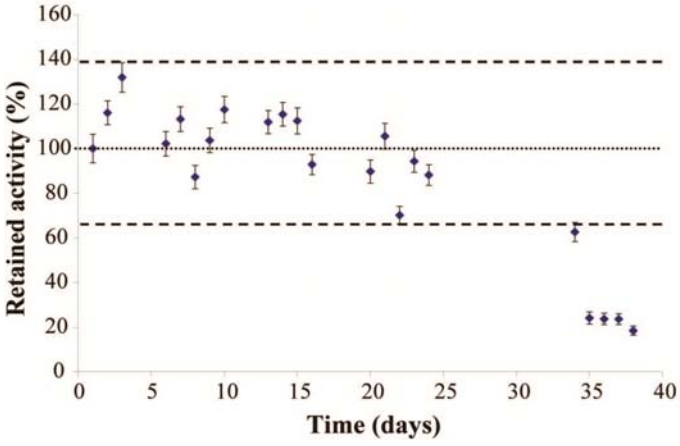

Fig. 5. Stability of the entrapped GOD in a Nafion matrix for the detection of 2.5 mM glucose. The applied potential was +550 mV. Data are represented as means ± SD.

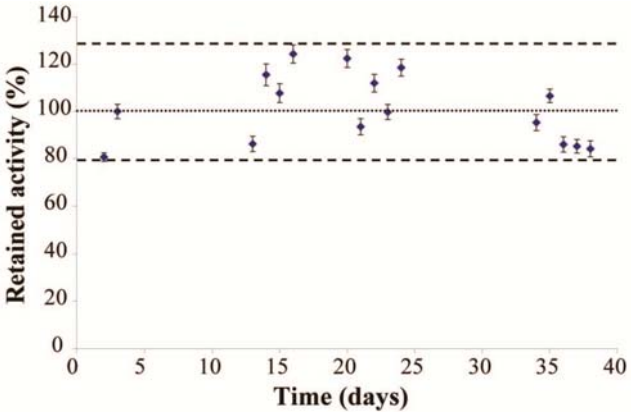

Fig. 6. Stability of the cross-linked GOD with glutaraldehyde for the detection of 2.5 mM glucose. The applied potential was +550 mV. Data are represented as means ± SD.

None of the three immobilization strategies highlights a specific trend over 25 days, but all the measurements oscillate around the 100 % of the retained activity. The difference is really evident after 30 days, where GOD cross-linked with glutaraldehyde shows the highest lifetime. In fact, the measurements are still around 100 % after 35 days, without any identifiable decay over the time. On the contrary, for the other two strategies of immobilization there is a clear decay after 24 days, with a consequent retained activity of around 16 % and 23 %, for adsorption and entrapment strategies, respectively.

The entrapment in Nafion (over the first 24 days) and the cross-linking (after the first 10 days) are the methods with the highest reproducibility, with an error around 14 %. GOD adsorbed on the electrode surface shows an error around 23 %, denoting lower reproducibility. The obtained values related to the retained activity over the time are quite similar with what was previously found in literature. Regarding the adsorption of GOD onto MWCNT, Wang *et al.* [10] found that for the detection of 1 mM of glucose, the retained activity after 25 days was about 97 %. Their sensor was quite similar to the one of the present work, since they adsorbed GOD onto grown MWCNT, without any other nanocomposite. The best result for the case of GOD immobilized without any other compound was obtained by Crounch *et al.* [17], where there was no apparent reduced activity of the sensor after 550 days. The immobilization strategy was instead different, since they developed a water-based carbon ink containing glucose oxidase and they stored the sensor in desiccated conditions, when not used. For the case of GOD entrapped in a Nafion membrane, Tang *et al.* [15] showed that the current response was still retaining the 73.5 % of the initial value after 22 days, coherently with what was found in the present work. For the case of enzyme cross-linking, the work of Kang *et al.* [18] showed a retained activity of about 92 % after 35 days and 85% after 50 days, in the case of 1 mM of glucose.

Table 1 reports the main features of biosensors developed with the three immobilization strategies. From the point-of-view of cell culture monitoring, the three presented techniques show an appropriate stability to be employed for this purpose. Although previous work presented good results in terms of lifetime for implantable sensors [19, 20], the research is not so focused on cell culture applications. For some biological studies, cell lines are not monitored for more than 40 days and there is a lack of research to develop suitable biosensors for this purpose. From the present research, we can conclude that glutaraldehyde cross-linking seems to be the most suitable to monitor septal neuron and stem cell lines, surviving for more than 40 days.

5. Conclusions

In the present work, we have investigated three different immobilization strategies to develop enzyme-based glucose biosensors using nanostructured electrodes for long-term cells monitoring.

Table 1. Resume of the main futures for the three immobilization techniques.

	Sensitivity ($\mu A\ mM^{-1}\ cm^{-2}$)	**Limit of detection (μM)**	**Linear range (mM)**	**Lifetime (days)**
Adsorption	10.24	97	0.1 – 6.5	24
Entrapment	8.18	59	0.2 – 7.5	34
Cross-linking	17.38	16	0.025 - 5	> 38

We have shown a comparison in terms of linear range, detection limit, sensitivity and long-term stability. For the case of GOD adsorbed onto the electrode surface, we found a linear range of 0.1 – 6.5 mM, a detection limit of 97 μM, and a sensitivity of 10.24 $\mu A\ mM^{-1}\ cm^{-2}$. In the case of GOD entrapped in a Nafion matrix, we found a linear range of 0.2 – 7.5 mM, a detection limit of 59 μM, and a sensitivity of 8.18 $\mu A\ mM^{-1}\ cm^{-2}$. For the GOD cross-linked with glutaraldehyde we observed a linear range of 0.025 – 5 mM, a detection limit of 16 μM, and a sensitivity of 17.38 $\mu A\ mM^{-1}\ cm^{-2}$. We also investigated the dependence of the sensor response related to Nafion quantity drop cast onto the electrode surface. We found that the more the volume of deposited Nafion, the lower the value of sensitivity. The best amount of Nafion results in 1 μl of 0.5 wt% Nafion. Finally, we investigated the lifetime of the three developed biosensors. All the three electrodes have a good stability over 25 days, with higher reproducibility in the case of GOD entrapped in Nafion and cross-linked with glutaraldehyde (errors around 14 %). In addition, the best result in terms of long-term stability is obtained with cross-linking, able to maintain stable measurements for more than 40 days. Further experiments will focus on other kinds of oxidases, in order to confirm the best strategy for enzyme immobilization, and to develop sensor array for the long-term monitoring of multiple metabolites in cell cultures.

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Contributions are invited on all aspects of research, development and application of the science and technology of sensors, transducers and sensor instrumentations. Topics include, but are not restricted to:

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- Digital, frequency, period, duty-cycle, time interval, PWM, pulse number output sensors and transducers;
- Theory, principles, effects, design, standardization and modeling;
- Smart sensors and systems;
- Sensor instrumentation;
- Virtual instruments;
- Sensors interfaces, buses and networks;
- Signal processing;
- Frequency (period, duty-cycle)-to-digital converters, ADC;
- Technologies and materials;
- Nanosensors;
- Microsystems;
- Applications.

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