Biosensors and Bioelectronics 24 (2009) 2702–2706

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Short communication

Glucose determination using a re-usable enzyme-modified ion track membrane sensor

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ARTICLE INFO

Article history:
Received 5 October 2008
Received in revised form 17 November 2008
Accepted 1 December 2008
Available online 7 December 2008

Keywords:
Glucose sensor
Etched tracks
Ion track membranes
Nanopores
Glucose oxidase

ABSTRACT

A novel concept for a glucose biosensor based on the enzyme glucose oxidase covalently linked to nanopores of etched nuclear track membranes is presented. This device can be used to detect physiologically relevant glucose concentrations between 10 μM and 1 M. The sensitive catalytic sensor can be made re-usable due to the production of diffusible products from the oxidative biomolecular recognition event. We have demonstrated both the simplicity with which this sensor can be produced and the stability of the enzyme embedded in the nanopores. We strongly believe that the approach could be expanded for different enzymes and variable configurations of sensing and catalysis using membranes with nanopores for biocatalysis and enrichment of substrates and products. This is the first time that such devices are being used for biocatalytic transformations.

1. Introduction

Latent ion tracks are zones of radiation damage brought about by electronic energy transfer mechanisms along the trajectories of highly energetic heavy ions impinging upon a solid. In polymers, this radiation damage often leads to chain scissioning along the ion tracks that promotes an enhanced etchability (Fink, 2004). As a consequence of such track etching, narrow and long parallel pores emerge—the so-called etched tracks—with diameters that are typically between 10 nm and 10 μm (depending on the etching time) and lengths of up to 100 μm, depending on the ion energy and the thickness of the irradiated polymeric foil (Fink, 2005).

Ion tracks are particularly suitable in biosensing applications because they have true nanometric dimensions. Ion tracks can confine chemical reactions in well-defined, pre-determined locations ensuring that their reaction products are highly enriched locally. If membranes containing such etched tracks are put in the path of ion currents flowing through a vessel, all the ions are subsequently forced to pass through the nanopores, electrically sensing any confined chemical reaction occurring there via changes in the pore's electrical resistance. The advantages of using conically etched ion tracks were pointed out by Siwy et al. (2005), in this connection, noting that the voltage drop in a conical nanotube is focused at its tip, thereby enhancing the sensor's detecting efficiency. Conically shaped, etched tracks emerge by etching latent tracks from only one side of a polymer foil.

Plenty of work has already been invested to study the transport processes in cylindrically and conically etched ion tracks. For example, ion selectivity has been found for gold-clad nanopores (Siwy et al., 2004), and cone-shaped nanopores rectify a transmitted electrical current which leads to an asymmetric current–voltage curve (*I–V*) (Siwy et al., 2002). Such diode-like *I–V* curves also indicate that there is a preferential direction for ion flow (Siwy, 2006) so that these pores act as ion pumps.

Ion tracks were demonstrated before as useful platforms for the transduction of specific biorecognition events (Siwy et al., 2005), where three different nanoporous systems were targeted at the detection of streptavidin, immunoglobulins and ricin by nanopore modification with appropriate respective recognition molecules. This type of sensor was based on the decrease of currents in an *I–V* curve due to pore blocking as a result of a single or a few recognition events, hence exhibiting unique detection sensitivity. Our approach allows a catalytically—hence continuously—operated sensor to be

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doi:10.1016/j.bios.2008.12.001

used. Using enzymes that produce soluble reaction products which diffuse away prevents the pore tip from becoming blocked, thereby rendering these sensors as re-usable.

We have set out to test the possibility to use the catalytic activity of enzymes and the resulting products as a possible transducable biocatalytic event in ion tracks, by using the enzyme glucose oxidase (GOx) as the catalyst for the oxidation of glucose in the presence of oxygen, for glucose biosensing purposes.

Many electrochemical glucose sensors have been developed along the years, due to their importance in medicine for the diagnosis of diabetes (Wang, 2008). One of the preferred approaches uses the enzyme GOx, which catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone which, in turn, hydrolyzes to gluconic acid. This oxidation reaction is accompanied by a change in pH and therefore, in the conductivity of the enzyme's immediate environment. It is on this change in conductivity that we are relying on as the basis for a signal in our biosensor.

In fact, it has already been suggested over a decade ago (Kuwabata and Martin, 1994) that ion track membranes with adsorbed GOx may be used for sensing purposes. However, it was shown that the catalysis of glucose took place by a Pt layer covering the membrane rather than the adsorbed GOx. We modified this approach such that now GOx is, in fact, the active sensing component, rendering the modified nanopore a more specific sensor for the desired analyte.

2. Experiments

2.1. Chemicals

Solutions were prepared using ultrapure water from NANOpure Diamond (Barnstead) water purification system. D(+)Glucose anhydrous, sodium dihydrogen phosphate, and di-sodium hydrogen phosphate dodecahydrate were obtained from Carlo Erba Reagents, Italy. Hydroxy-2,5-dioxopyrrolidine-3-sulfonicacid sodium salt (sulfo-NHS), N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 2-(N-Morpholino)ethanesulfonic acid hydrate (MES hydrate), 30% hydrogen peroxide solution, glucose oxidase from *Aspergillus niger* (GOx) (E.C. 1.1.3.4), and horseradish peroxidase (HRP) (E.C. 1.11.1.7) were purchased from Sigma–Aldrich, Israel. Dulbecco's phosphate-buffered saline was purchased from Biological Industries, Israel. The chemiluminescence substrate Immun-Star™ HRP Luminol/Enhancer was purchased from Bio-Rad Laboratories Inc. Hercules, CA. NaCl was purchased from Bio-Lab Ltd., Israel. Sodium hydroxide was purchased from Gadot, Israel. Hydrochloric acid 32% was purchased from Frutarom Ltd., Israel.

2.2. Ion track preparation

12-μm thick polyethylene terephthalate (PET, Melinex) foils of Russian provenance were irradiated by 250 MeV Kr ions up to fluencies of 4×10^6 cm^{-2} at the swift heavy ion accelerator at the Flerov Institute, INR, Dubna. The foils were etched from one side at ∼40 °C by 0.2 M NaOH, while the other side was kept in contact with 0.2 M HCl to stabilize the pore's tip after breakthrough by neutralization of the etchant.

After rinsing with double de-ionized water, the nanoporous foil is ready to uptake the enzyme in the nanpores. Initial experiments revealed that the negatively charged GOx did not adhere to the negatively charged walls of the nanopores. Also, the use of a phosphate buffer to promote GOx adherence produced insufficient results. In these cases, however, the enzyme originally showed the expected activity, after which it was apparently gradually desorbed from the nanopores, and therefore, enzyme activity decreased steadily.

2.3. Sensor preparation

Preparation of the membrane for the covalent attachment of GOx was performed as follows: the etched membrane with exposed carboxyl groups was washed with MES activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) to prepare it for subsequent attachment of the enzyme's amine groups. Then EDC (2 mM) and sulfo-NHS (5 mM) in a volume of 3 ml were added to the membrane and allowed to react for 15 min at room temperature. After the reaction the membrane was washed with activation buffer again and a GOx solution of 0.5 mg/ml in activation buffer was added. The enzyme coupling reaction was performed for 1 h at room temperature. The membrane was then washed three times with phosphate buffer saline (PBS 0.1 M, pH 7.0) to remove unbound enzyme. The modified membrane was used immediately and all glucose dilutions were prepared in PBS 0.1 M, pH 7.0. An optimal GOx concentration for modification was determined according to the rectification ratio (*vide infra*). pH of operation and temperature for sensor preparation were predetermined according to previously known procedures in the fabrication and characterization of GOx-based glucose sensors (Heller and Feldman, 2008).

2.4. Ion flow measurements

Upon nanopores stepwise modification and the addition of glucose, current voltage curves were measured in a custom-made vessel in a volume of 3.0 ml using a 2-channel USB-PC Oscilloscope PCSU1000 and a Function generator PCG10a, both of which are from Velleman, Belgium. Electrodes that were used for measurements were 1 cm long silver wires with 0.5 mm diameter placed 1 cm away from the sensor foil.

2.5. Hydrogen peroxide content determination

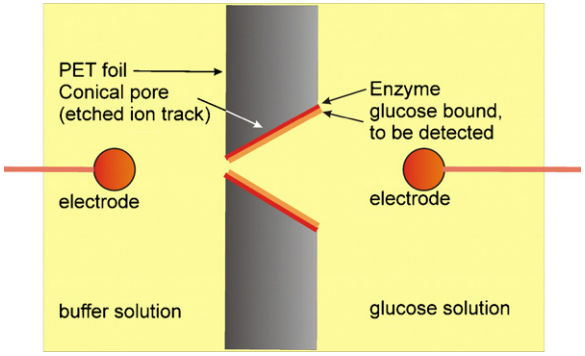
Glucose solutions were collected after the reaction with GOx to measure hydrogen peroxide concentrations to determine whether they correlate with the added glucose concentrations. A BioTek Synergy HT plate reader (BioTek Instruments, Inc., VT, USA) was used to monitor the chemiluminescent signal and to process data in real time. The chemiluminescent assay was performed in a 96-well plate in a total volume of 200 μl containing 1 U/ml HRP, 10 μl enhanced luminol reagent solution and 180 μl of collected glucose sample. Over the course of 10 min, chemiluminescence readings for each sample were integrated for 1 s with time intervals of 26 s between readings. The same experiment was performed with known hydrogen peroxide concentrations to build a calibration curve. Peroxide concentrations in glucose samples were calculated based on the calibration curve.

3. Results

3.1. Biosensor construction

Upon etching of the PET foils 4×10^6 cm^{-2} conical nanopores were formed with the dimensions of $\sim 1.0 \pm 0.1$ μm at the wide end and $\sim 30 \pm 20$ nm at the tip, according to previous characterizations that were performed by us using ion transmission spectrometry and scanning electron microscopy (Petrov, 2004).

Preparatory steps and the corresponding chemical reactions were permanently monitored via the changes in track characteristics. For that purpose two electrodes were inserted into the measuring vessel, which was separated by the sensing membrane into two compartments (Scheme 1). A sinusoidally shaped AC voltage of up to $5\,V_{peak-peak}$ operating at 1 Hz was applied to the electrodes. Both the voltage and the transmitted ion current were recorded by an oscilloscope.



Scheme 1. General scheme describing the detection scheme and modified polymer.

Based on the current/voltage characteristics obtained for the different preparatory steps, the most pronounced diode-like curve, being characterized by a rectification ratio r (defined here as the absolute value of the current ratios at +500 mV and −500 mV) around 9.5, was observed immediately after track etching (Fig. 1A and B). A diode like I–V curve indicates that there is a preferential direction for ion flow (Siwy et al., 2003). The addition of an EDC/sulfo-NHS solution markedly reduced the current/voltage readings by a factor of about 25 (Fig. 1C and D), with a decrease of r towards 4.2. This may signify a reduction in the number of surface charges on the ion track walls, since carboxylate ions from the polymer are mainly covalently bound to sulfo-NHS in the presence of EDC. Conjugation of the enzyme to the track walls (Fig. 1E and F) causes another reduction, roughly by a factor of 5, due to a possible narrowing of the ion pore, but the rectifying characteristic is still somewhat preserved with a rectification ratio of 1.4.

However, the addition of small amounts of glucose (Fig. 1H) altered the rectifying characteristics to near-Ohmic (rectification ratio $r = 0.94$ for 5 mM glucose; ideally Ohmic: $r = 1$), indicating that all surface charges on the tracks had been compensated. Simultaneously, the transmitted ion current again increased, indicating that the expected catalytic enzymatic reaction did indeed take place. The small hystereses in all curves stems from the high dielectric constant ($\varepsilon = 80$) of the water in the tracks, which leads to a minor capacitive contribution, even at the low sweeping frequency of 1 Hz that was used here. Addition of higher glucose concentrations (Fig. 1G) leads to a slight inversion of the rectification (e.g. $r = 0.62$ for 10 mM glucose), for yet unknown reason (for a graphical summery of rectification ratio changes upon track modifications and a possible explanation for the inversion of rectification phenomena, please see supplementary data section figure SD1).

3.2. Glucose dependency calibration curves

To establish the sensor's calibration curve, the I–V relations were recorded for different glucose solutions added to the sensing device.

At each step the sensor was allowed to adjust for ~5 min before recording, after which solutions where collected to determine hydrogen peroxide concentrations using HRP luminescence assay.

The calibration curves of all successfully produced biosensors could be compared with each other because their current/log

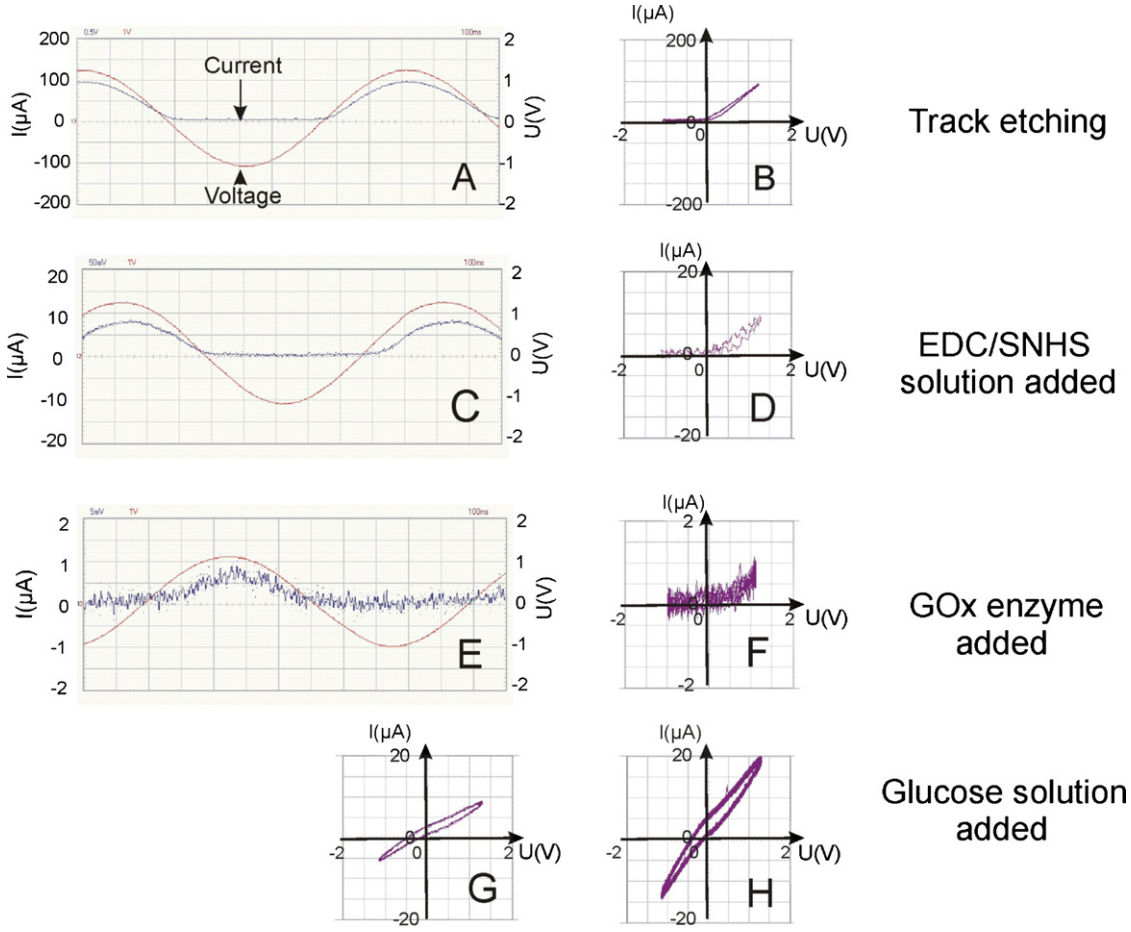


Fig. 1. Current/voltage correlations for the different stages of glucose sensor preparation: (A and B) after track etching; (C and D) after adding EDC/sulfo-NHS solution; (E and F) after addition of enzyme; (G) after adding 5 mM glucose solution; (H) after adding 10 mM glucose solution. The lowest currents are affected by electronic noise. In some cases, e.g., figures F, G, and H, several sweeps were overlaid to control the straggling of individual data. The measurements refer to an applied sinusoidal voltage of 1 Hz.

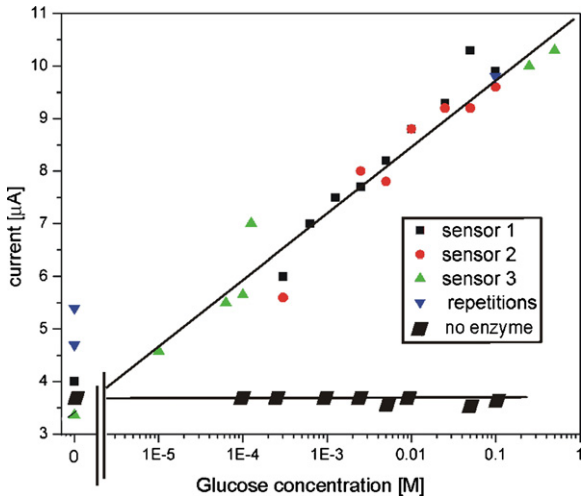


Fig. 2. Performance comparison of three identically produced track-based glucose detectors against a calibration curve *I* (+5 V) vs. glucose concentration. The calibration curves of sensors 2 and 3 and of the membrane without enzyme were normalized to that of sensor 1 by multiplying the currents of the sensors by 2.0 and 0.64, respectively. Current response to a pure buffer solution (i.e., glucose concentration = 0) is added on the left side. The standing triangles show for sensor 1 the accuracy within which a measuring cycle can be repeated.

(concentration) plots all exhibited the same slope (Fig. 2). As their absolute values differed within about one order of magnitude due to the different etch track diameters (depending on the time after pore breakthrough when the etching was interrupted and possibly based on different enzyme coverage, which is related to variations in the chemical preparation steps), they have to be normalized to each other. Furthermore, the graph contains some values that were measured repeatedly to estimate sensor reproducibility. Within the measured interval of nearly five orders of magnitude (from 10 μM to 0.5 M), the sensors exhibited logarithmic behavior in the calibration curves. Individual measurements deviate from the calibration curve by less than a factor of 2.

To prove that the calculated calibration curve exclusively reflects glucose oxidation by GOx and not the non-specific oxidation of glucose, these measurements were repeated with membranes that were not modified with GOx but that were otherwise prepared under the same conditions. No change was observed in the transmitted ion currents of these membranes (Fig. 2, parallelograms).

Another hint that the measured current increase upon addition of glucose to the buffer must derive solely from the enzymatic reaction is found in Fig. 1E and F. The near vanishing of the nanopore rectification upon enzyme attachment indicates that surface charges on the nanopore walls have been practically used up, hence cannot contribute to any surface conductivity along the pore wall (which otherwise could have been an alternative explanation for the effect found here).

3.3. Sensor response time

To test sensor reproducibility the applied glucose concentrations were altered between 0.1 M and 0 M. Whereas the measurement for the highest concentration was repeatable, the values for near zero concentrations showed slight increases in current possibly due to contamination by traces of glucose that remained in the vessel despite repeated washing steps. Typical detector response time to new concentrations was less than 20–30 s for small concentration differences. The response time appears to increase, however, as consecutive concentrations differences become larger and with the decrease in enzyme stability over time (for a graph demonstrating sensor response time, please see Supplementary data Fig. SD2).

3.4. Hydrogen peroxide evolution validation

According to the reaction between GOx and glucose in the presence of oxygen, hydrogen peroxide should be produced in a 1:1 ratio with oxidized glucose. Hence, we measured the hydrogen peroxide content using an independent enzymatic luminescence assay (using horseradish peroxidase). The catalytic reaction was run at several glucose concentrations, and corresponding solution samples were collected from the sensing device. Indeed, the hydrogen peroxide content correlated within an order of magnitude (within the limits of the luminometer) with the glucose concentrations that were introduced into the sensing device (data not shown).

4. Discussion

One should bear in mind that due to the asymmetric electric field distribution as a consequence of the conical pore shape and the surface charges, a uni-directional flow of electrolytes only is possible. However, once the surface charges are compensated by enzyme attachment, this effect vanishes so that our biosensors are assumed to be neither preferential for some ion flow direction, nor cation-selective. This is advantageous, as it avoids undesired nanopore-specific side effects during sensing (for further discussion of the regime and kinetics of flow inside ion tracks please see supplementary data).

In our experiments, we cannot exclude that also charged gluconic acid (i.e. the gluconate ions) carries current, it is also well established (Tam et al., 2008) that the biocatalytic transformation of glucose reduces the pH value. Therefore, possibly both ions (gluconate and H^+) contribute to the measured current changes.

The calibration curves of several produced glucose sensors had been found to be parallel, just differing by factors of $\sim 2 \pm 1$ or less from each other, so that they could be normalized to one master curve (for the latter, the mean of all measured curves was chosen, Fig. 2). The deviations in the currents for a given glucose concentration, as measured with different sensors, are thought to stem essentially from slight variations in their pore tip diameters during etching thus allowing for somewhat different electrical currents to be transmitted. The correlation of the detected changes in conductivity is understood to result essentially from local changes in the pH value in the confined region of the track tip due to the presence of the biocatalyzed chemical reaction products. Therefore we conclude that specially adjusted pore tip diameters are not required; any tip size will suffice provided that the pore diameter is comparable to the thickness of the electrical double layer to enable reasonable reaction product confinement.

We demonstrated that glucose is indeed being oxidized by GOx and not by any of the other components of the device. We also quantitatively demonstrated the presence of the oxidation product using the independent approach of hydrogen peroxide-dependent luminescence. Furthermore, luminescence measurements were less sensitive to hydrogen peroxide than was the enzyme-modified ion track sensor for the presence of glucose.

The biosensor presented here is small and lightweight, consisting of a 12 μm thick polymer foil with an area of $\sim 1\ cm^2$. To measure moderate glucose concentrations, it could be reduced in area by one or two orders of magnitude.

The current/voltage characteristics in the glucose-measuring mode approach linearity (Fig. 1G and H), indicating direct correlations between the applied voltage and the current signal, and, therefore, also between the applied voltage and glucose detection sensitivity. Although the results of this work are restricted to $5\ V_{peak-peak}$, it was found that the voltages can be increased at least up to $10\ V_{peak-peak}$ without affecting enzyme operation or stability.

The sensitivity range (10 μM to 1 M) of the glucose detector proposed here is comparable to those reported in the literature. We

found good linearity between the measured ion current through the sensor and the applied glucose concentration in the lin/log plot over the whole measured range. Capability is still slightly limited when measuring cycles that alternate between very high and very low concentrations.

We could perform at least a dozen measurements per sensor before a marked deterioration sets in. The sensor proved to be useful for at least 1 day of operation at room temperature, and for more than a week if kept at 4 °C in between measurements.

5. Conclusions

Primarily a feasibility study to demonstrate the usefulness of re-usable ion-track-based biosensors; this work showed that ion-track-based glucose sensors can be easily created. Furthermore, they show good sensitivity, they cover the range of medical applications, and they can be reused at least 10 times. Pre-measurement system adjustments usually require less than a minute. If the measurements are performed carefully, with the exception of very high and low concentrations, accuracies in the order of ±10% can be obtained for a given sensor, see the central part of the calibration curves of Fig. 2. Different sensors vary in their responses from each other by factors of up to 2 ± 1.

This study also shows that track-based biosensors with other enzymes (e.g., alcohol dehydrogenase, lactate dehydrogenase), even in configurations of several enzymes arranged consecutively (e.g., acetylcholine esterase with choline oxidase), can be similarly developed. The positive results reported here encourage the continued development of more electronically refined biosensors by combining enzyme-loaded tracks with metal-oxide-based semiconductors and/or silicon for the direct integration of the sensors into electronic devices (Fink et al., 2004).

Acknowledgments

We are obliged to Drs. P. Apel and A. Schulz for leaving us some ion-irradiated polymer foils. Further, D.F. is grateful to BGU, and A.K. thanks the HMI (now: HZB) for travel support. L.A. acknowledges the support of the Edmond J. Safra Center for the Design and Engineering of Functional Biopolymers in this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.12.001.

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