ARTICLE TYPE

Ultratrace Detection of Glucose with Enzyme-Functionalized Single Nanochannels

Guanglei Hou,[a] Huacheng Zhang,[a] Ganhua Xie,[a] Kai Xiao,[a] Liping Wen,[a] Shuhong Li*[b], Ye Tian*[a] and Lei Jiang[a]

A sensitive nano-device for D-glucose detection is prepared by modifying GOx enzymes into a single conical polymer nanochannel. Current-Voltage (I-V) characterization suggests
10 **that the nano-device could response to D-glucose with concentration down to 1 nM (10^{-9} mol L^{-1}) rather than its enantiomer. Moreover, the nano-device behaves good reproducibility and specifity to D-glucose and will provide ideal candidate for commercialized non-invasive blood**
15 **glucose meters in the future.**

Introduction

The emerging smart nanochannels have attracted great interest and have been applied to construct various kinds of micro/nano-devices, such as biosensor.[1-5] Since the biomolecules could be
20 anchored at high specific surface area in the nanochannel without recession of bio-activity,[6, 7] the nano-scaled aperture of single nanochannel is comparable to the size of modified molecules and analytes,[8] and the shape could also be adjusted to output the electric signals in a discernible asymmetric form,[9, 10] the
25 nanochannels become preferential choice for extremely sensitive detection. Recently, smart nanochannels have been widely investigated by immobilizing functional aptamers,[11-14] enzymes[15-17] or peptides[18, 19] on to the inner wall of the nanochannel for sensing. However, most previous researches are focused on the
30 transient blockade of aperture of nanochannel that induced by the "weak interactions" between modified molecules and analytes, such as metal ion-biomolecules affinity,[20, 21] electrostatic adsorption[22, 23] and so on. Little attention has been paid to realize the sensing performance by changing the surface chemical
35 properties via "strong interactions", like enzymatic catalytic reactions.

Nanomaterials (nanoparticles, nanotubes, nanofibers and their nanocomposites) enhanced the performance of glucose biosensors, and achieving more superior sensitivity. Sun et al.
40 reported a pH gated glucose responsive nanochannel which modified with 3-aminobenzeneboronic acid as the glucose conjugation sites.[24] Glucose as low as 0.1 μM could be detected by this device. Li et al. immobilized GOx enzymes onto electrodeposited Pt nanoparticles chitosan composite film, limit
45 of detection with 0.4 μM can be achieved.[25] Carbon nanofibers were employed by Islam et al. to construct a mediator free amperometric bienzymatic glucose biosensor, and the limit of

detection is 0.4 μM.[26] Parviz reported a glucose biosensor that nafion films composed of MWCNTs and gold nanoparticles were
50 deposited on the glassy electrode could reach the goal of 0.03 μM detection limit.[27] The blood glucose sensors undergo great improvement from implanted devices to more superior non-invasive equipments which are expected to operate conveniently and in comfort.[28] Compared with the implanted devices, non-
55 invasive blood glucose meters have to choose perspiration, saliva and tears as the analytes. Unfortunately, glucose is extremely insufficient in these exudates therefore it is urgent to develop the ultratrace detectable glucose biosensors.[29]

In this work, we report a nano-device for ultratrace glucose
60 detection based on the glucose oxidase (GOx)-modified single nanochannels (GSNC). The single conical nanochannel was prepared in 12 μm thick polyethylene terephthalate (PET) foil with single damaged track by asymmetric chemical etching method. The diameter of the large opening (base side) was ~ 1.3
65 μm (Figure S1) and the size of small opening (tip side) was caculated to be 30 nm by the classic electrochemical method.[30] As shown in Scheme 1a, the exposed – COO^- groups on the channel surfaces can be bound with GOx enzymes by the coupling reaction.[31] Glucose oxidase has been profoundly studied
70 due to their excellent oxidation ability to D-glucose. D-glucose can be oxidized to D-gluconate and proton by glucose oxidase in the present of oxygen molecules (Scheme 1b). The functionalized nanochannel shows excellent specific recognition capability to D-glucose, which was manifested via the changes of the
75 transmembrane ionic current. Once D-glucose get access to the GSNC, the enzymatic reaction will change the transmembrane ionic current both magnitude and polarity. Detection of the D-glucose down to 1 nM can be achieved by this functional nanochannel. To our best knowledge, the the detection limit was
80 the lowest among the present nanomaterials based glucose detectors. Moreover, the GSNC could response to D-glucose rather than its enantiomer in the form of visualized electric signal, and it behaved good reproducibility in 5 repetitions. The novel GSNC device will supply a considerable candidate for
85 commercialized non-invasive blood glucose meters in the future.
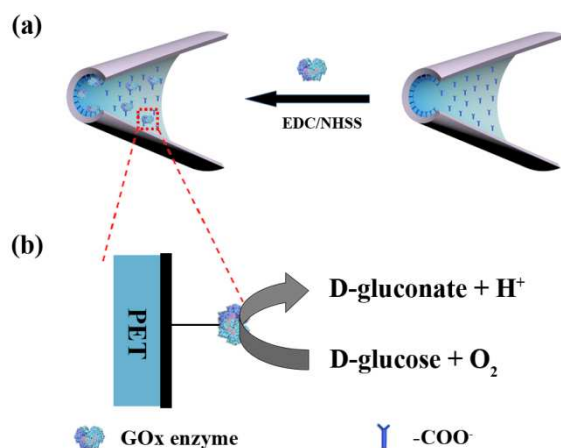
Scheme 1 Schematic representation of an ultratrace D-glucose recognized single conical nanochannel. (a) Covalently linkage of the glucose oxidase (GOx) enzyme molecules to carboxyl groups in a single conical
5 nanochannel by carbodiimide coupling chemistry. (b) Scheme of the enzymatic reactions occurred in the GOx-modified single conical nanochannel. Acidic productions were generated in the present of oxygen molecules. The scheme is not drawn to scale. The space filling model of GOx was cited from Protein Data Bank (PDB: 1CF3).

Results and discussion
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For the purpose of characterizing the enzyme modified nanochannels, I-V measurements were carried out respectively before and after GOx modification. It is clear that the naked PET single nanochannel showed a slightly rectified I-V characteristic
15 due to negatively charged surface and conical shape design. A significant decrease of ionic current can be seen in Figure S2 after GOx modification. Two reasons can be adopted to interpret the alterations of ionic current. Firstly, the steric hindrance aroused from anchored GOx molecules whose average size were
20 estimated to $8×7×5.5$ nm^3 in solutions.[32] Secondly, screening of surface charge after modification decreases the transfer and rectifying ability of channel to contra-ions. Steric hindrance and screening of nanochannel surface charge exhibit synergistic effect in decreasing ionic current after the GOx enzymes were
25 immobilized on PET nanochannel surface. In a word, the distinction between these two I-V curves confirmed the success of enzyme modification. Furthermore, XPS characterization also confirmed the achievement of GOx modification on PET nanochannel surface by emerging of the nitrogen peak after GOx
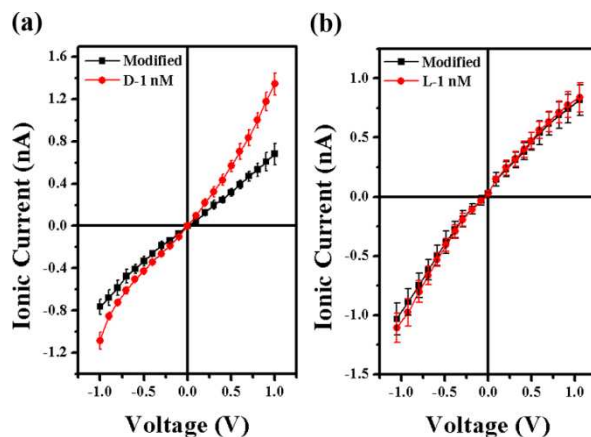30 modification.

Fig. 1 I-V characteristics of the GSNC filled with test solution (0.1 M KCl + 0.05 M PB, pH 5.5) containing 1 nM (a) D-glucose and (b) L-glucose. The diameter of the nanochannel tip side without GOx
35 modification was about 30 nm. The applied external bias was from -1 V to +1 V, and anode face the base side of the channel in our electrode configuration.

Sensing performance of the GSNC system was examined after modification of enzymes was achieved satisfactorily. Figure 1
40 shows the I-V curves of nanochannel after GOx modification in the present of ultratrace D- and L-glucose respectively. The obvious increase of ionic current could be detected even though the D-glucose concentration was as low as 1 nM, and the difference of ionic current is about 0.7 nA when the applied
45 voltage is +1 V (Figure 1a). It is worth noting that the increase of ionic current at the positive bias is much larger than that at the opposite interval. The phenomenon could be ascribed to the variations of surface chemistry induce by the "strong interaction" between modified enzyme molecules and analytes. The polarity
50 of surface charge was inversed from negative to positive by the low pH environment after enzyme catalytic reactions, so that the anions were selectively transport from the tip to the base side at the positive bias. The modified nanochannel exhibited subtle resolution than that of volume exclusion mechanism owing to the
55 surface charge modulation. This result is similar with Dekker's work which modified GOx enzymes onto single wall nanotubes (SWNT).[33] It suggests that the nano-device behaves high sensitivity and exhibits excellent responsive signals to D-glucose with concentration down to nanomolar. However, no significant
60 variation was observed when L-glucose existed in the GSNC (Figure 1b). This result is in accordance with the previous reports about the conclusion that GOx is unable to catalyse L-glucose. The negligible current variation in I-V curves is probably attributed to the turbulence throughout the membrane. The
65 experimental results confirmed that such a nano-device also behaves excellent chiral recognition ability.

To ensure the increase of ionic current is not due to the conductivity of glucose, naked conical nanochannel without GOx-modification was also tested by adding D-glucose solutions.
70 According to Figure S3, no obvious responsive current signal can be seen. It implies that the presence of GOx enzymes and enzymatic reactions is the key for nanochannel to produce electrical signals.
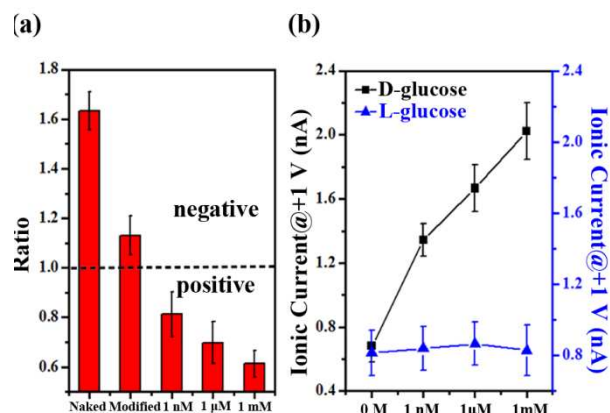
Fig. 2 (a) Ratios of single conical PET nanochannels recorded at different test conditions. The ratios of nanochannels were less than 1 without D-glucose, implied negatively charged inner channel surface. On the other hand, ratios fell below 1 after D-glucose addition implied positively charged inner channel surface. Acidic products of enzymatic reaction drove down the pH value of nano-confinement in the channel chamber, thus protonated amino groups on GOx enzymes leading to reversal of surface charge polarity. Ratio = │ L_{1V} │ / │ I_{+1V} │ . (b) Current-concentration characteristics of the GSNC in the present of different concentrations of D- or L-glucose at +1 V.

The ratio is usually calculated as the absolute value of quotient of ionic currents when the applied voltage was in the same value but opposite polarities. Since the conical nanochannel behaves like diode to rectify the ion flux, current rectification is considered as a "wind vane" of the polarity and distribution of nanochannel inner surface charge. Figure 2a summarized the ratio changes at all conditions to compare the variation of nanochannel sensing ability. The ratio of naked PET nanochannel is 1.7 before GOx modification, indicating that the channel surface was negatively charged due to deprotonation of carboxyl groups under our electrode configuration and neutral pH solution conditions. At the same time, asymmetric distribution of surface charge generated "potential trap" at the tip side of the conical channel which is also helpful to transport ions asymmetrically. Therefore, the detected cationic transmembrane current is larger at the negative bias than the anionic current at positive bias. Once GOx enzymes were modified on the channel, the ratio decreased to 1.1 and the rectified ability of modified nanochannel seemed vanished due to the reduction of surface charge density. It can be attributed to partial shield of surface charge by GOx enzymes. Meanwhile, compare with carboxyl groups on the naked PET channel, the isoelectric point of GOx is much closer to the pH of circumstance. It means that the GOx bears more sparse negative charge and the rectify ability decayed somehow after modification. Ratio became less than 1 after D-glucose was added, namely reversal of current rectification direction. It means that the polarity of the single nanochannel surface charge has inversed from negative to positive. As the concentration of added D-glucose increases, the ratio decreases continuously. This observation could be inferred from the two-steps catalytic redox reaction that acidic species were yielded in the presence of oxygen molecules as the electron acceptor.[34] We can reasonably speculate that the acidic productions were capable of lowering down the pH value of the test solution existing in the nanochannel confinement. Control experiment in the macroscopic

dosage confirmed the decrease of system pH (Figure S5). Positively charged channel surface was obtained because the protonation of enzyme residues' amino groups when the local pH value below GOx's isoelectric point. The reciprocal of ratio at 1 nM is larger than ratio after modification suggests that the density of surface positive charge is higher than the density of negative charge in the same solution (pH 5.5), namely the anionic transportation ability of GSNC is stronger than that of cationic. Zeta potential at flat PET surfaces was measured as the evidence of pH induced membrane channel charge alteration (Figure S7b). Zeta potential of the GOx-modified flat PET film reversed from negative to positive at low pH condition. This conclusion is consistent with the results shown in Figure 1a that the transmembrane ionic current of D-glucose added nanochannel was larger than the GSNC at the same voltages. As more D-glucose was added, more acidic production was produced leading to more significant pH decrease which pulled down the ratios subsequently. Figure 2b describes the current changes of the modified nanochannel at +1 V bias upon the addition of D-glucose or L-glucose with different concentrations under the same conditions. The ionic currents increased gradually with increasing D-glucose concentration (Figure 2b, ■ black) whereas the currents almost kept in constant for L-glucose in the same concentration range (Figure 2b, ▲ blue). This experiment insured that the nano-device remained ideal identification at the glucose tests with ultratrace concentration.
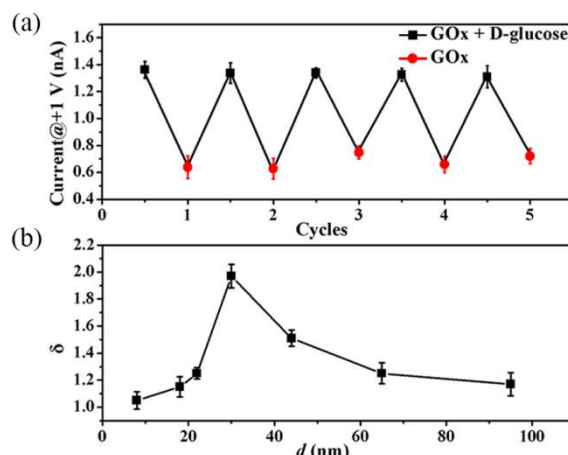

Fig. 3 (a) Reversible variation of the transmembrane ionic current measured at +1 V in 0.1 M KCl containing 500 mM PB. Major transmembrane electric signal was observed in the presence of 1 nM D-glucose (■, black). Meanwhile, minor ionic current indicated the absent of D-glucose (●, red). Clear distinction remained after 5 cycles of alternative measurments. The solutions were exchanged manually. (b) The dependency of sensing capability δ upon nanochannel tip diameters d. The optimal δ was observed at $d = 30$ nm. The δ of both smaller and larger nanochannels decreased due to steric hindrance and weak double layer control respectively.

The test of reproducibility of detecting D-glucose was carried out to evaluate the reusability and stability of the GSNC system. Figure 3 depicts the reproducibility of the nano-device using the same electrolyte in the presence or absence of trace D-glucose substrate. The reversible variation of the transmembrane ionic current at +1 V keeps a clear distinction about 0.7 nA after 5 cycles suggest that the GSNC exhibited a well-defined reproducibility. It is reasonable to conclude that such a nano-

device performs stable and reliable responsive signals, can be used for real time continuous glucose monitoring.

For the purpose of probing the effects of nanochannels diameter to sensing performance, a series of conical nanochannels with different diameters *d* were employed. As shown in Figure 3b, enzyme modified nanochannel that tip diameter is 30 nm showed the best sensing capability δ (δ was defined as I $_{(1 nM)}$ / I $_{(0 M)}$ at +1 V bias). The sensing capability δ decreased with the gradual deviation of d = 30 nm. The swelling enzymes are difficult to enter the lumen of the nanochannels that d < 30 nm. For the nanochannels that d > 30 nm, d is too larger than the electric double layers that both exits at the upper and bottom nanochannel surfaces. Thus, the double layer controlled ionic current acconts for a small percentage of transmembrane current.

Conclusions

In summary, we have constructed a nano-device that could detect ultratrace glucose by immobilizing GOx enzyme molecules onto the single conical shaped nanochannel surfaces through firm amide linkage. The conical single channel in the nano-scale experienced variation of surface chemistry that induced by enzyme catalytic reactions behaves good reproducibility and specificity to D-glucose with the detect limitation to 1 nM. Such a biomimetic nanochannel will further develop non-invasive blood glucose sensors and meets the demands for detecting ultratrace D-glucose in the field of clinical therapy, ferment monitoring, and food analysis.

Acknowledgements

Notes and references

a Beijing National Laboratory for Molecular Science (BNLMS), Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Science, Beijing 100190, P. R. China; E-mail: tianyely@iccas.ac.cn
b School of Science, Beijing Technology and Business University, Beijing 100048, P. R. China; E-mail: lish@th.btbu.edu.cn

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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