Preparation of photoluminescent enzymatic nanosensors for glucose sensing

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Abstract

A novel photoluminescent glucose nanosensor was facilely prepared by coupling glucose oxidase (GOx) with poly-L-lysine coated oxygen nanosensors via a glutaraldehyde-mediated Schiff-base reaction. The GOx molecules residing on particle surface catalyzed glucose with the expense of oxygen, which was detected by the sensing particle core incorporated with the reference dye coumarin 6 and oxygen probe Pt(II)-*meso*-tetra(pentafluorophenyl)porphine. The proposed glucose nanosensors (~150 nm in hydrodynamic diameter) had a quick response time varied from less than 2 minute to 4 minute. Glucose calibration was performed with ratiometric photoluminescence and time-resolved fluorescence (TRF) respectively, and a series of calibration plots were constructed according to determination time. In comparison, the ratiometric method resulted in wide dynamic range (e.g. 2-10 mM) and high limit of detection (~1-2 mM), while the TRF mode gave narrow dynamic range (e.g. 1-6 mM) with low detection limit (~ 0.1-0.2 mM). Finally the enzymatic glucose nanosensors were tested in human serum samples with a TRF microplate reader.

Keywords: Glucose; Enzymatic nanosensor; Oxygen; Time-resolved fluorescence; Ratiometric photoluminescence

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1. Introduction

As a primary energy source in most organisms and an important metabolic intermediate, glucose is indispensable to all living creatures. Determination of glucose concentration in fluids plays a big role in biochemistry, clinical chemistry and diagnosis of diabetes particularly. [1, 2] Tremendous amount of biosensor for glucose has been developed to this end. [3-6] More recently, a great number of nano-systems have emerged as bioprobes for glucose sensing with extra bonus of improved sensitivity due to the increasing surface area of nanoparticles (NPs) and nanoscale sensibility. [7] Based on electrochemical mechanism, for instance, nanoporous materials (eg. Pt thin film [8] and PtPb networks [9]), nanotubes (ZnO arrays [10] and carbon nanotube) [11] and hybrid nanostructures (Pd NPs-carbon nanotube [12] and Cu NPs-graphene [13]) have been fabricated to assay glucose. Unfortunately, these sensing nanosystems may suffer from interference from electroactive species like urea, ascorbate and urate, or absorptive species, and also are difficult to realize non-invasive detection which is much desirable for *in vivo* measurement.

Optical glucose nanosensors, in contrast, are capable of non-intrusive measuring while immune from the common interfering species. Several types of nanoprobe have been constructed by using of noble metal NPs, semiconductor quantum dots (QDs) or carbon dots, which are further conjugated with bioactive molecules such as concanavalin A, [14] boronic acid [15-18] and glucose oxidases (GOx). [19-22] With the presence of glucose, the sensing nano-systmes either aggregate to give rise to shifted plasmonic absorption or weakened fluorescence, or biocatalytically generate H₂O₂ to quench fluorescence of QDs. It is noticed, however, that the widely used detection approach in bulk enzymatic sensor - oxygen-sensitive fluorescence (from oxygen probe) cooperated with GOx - is rarely adopted to build glucose nanosensors. [23] As far as we are concerned, only one such glucose nanosensor has been reported so far, which was constructed by incorporating GOx and a phosphorescent oxygen indicator sulfo-Ru(dpp) into polyacrylamide nanoparticles. [24] Although the phosphorescent nanosensors are sensitive to glucose, the encapsulation of GOx might retard the catalytic oxidation of glucose.

Previously we had prepared poly-L-lysine (PLL) coated oxygen nanosensors that based on oxygen-sensitive phosphorescence. [25-27] The nanosensors can facilely conjugate with functional groups such as (3-carboxypropyl) triphenylphosphonium through surface amino groups on PLL shell. [28] It naturally occurs to us that coupling GOx molecules with PLL-coated oxygen nanosensors would result in a fluorescent glucose nanosensor. Herein, a novel glucose nanosensor is proposed in this way, which consists of (i) a PLL-coated sensing particle core doped with the reference dye coumarin 6 and oxygen probe Pt(II)-*meso*-tetra(pentafluorophenyl)porphine and (ii) an outer layer of GOx for glucose. The resultant nanosensors, with a hydrodynamic diameter of ~150 nm, are highly sensitive to glucose in both photoluminescence intensity and lifetime, and respective calibration curves are plotted with different determination times. The functionality of glucose nanosensors are tested in human serum samples by using of a time-resolved fluorescence based plate reader, and reasonable results are derived.

2. Material and methods

2.1 Materials

Polystyrene (PS), poly-L-lysine (PLL), coumarin 6 (C6) and Pt(II)-*meso*-tetra(pentafluorophenyl)porphine (PtTFPP) were purchased from Sigma-Aldrich. Dodecyltrimethoxysilane (DTS), glucose oxidase (GOx from

Aspergillus niger, 100 units/mg) and glutaraldehyde (50%, GA) were from Jiaxing Sicheng Chemicals Co., Ltd (China), Aladdin (China) and Sinopharm (China), respectively. THF, D-glucose anhydrous (99%) and mineral oil (pure) were obtained from J&K (China). All reagents were used as received without further purification. Deionized water (DI) was used in all experiments.

2.2 Synthesis of PLL-coated photoluminescent oxygen nanosensors (PLL-NPs)

In a typical synthesis, C6, PtTFPP, PS and DTS were dissolved in THF, in a 2:1:47:50 weight ratio, and at a total concentration of 200 ppm. Then, 500 μL of mixed solution was rapidly injected into 8 mL water containing 0.16 mg PLL (pH 9, adjusted by ammonium hydroxide) under ultrasonication. The solution was then left standing for 2 h, and afterwards dialyzed against water for 24 h to remove the organic solvent. Finally, the NPs suspension was centrifugated (16000 r/min, 30 min.) to get rid of surplus PLL and other small molecules, and redispersed into DI water at a concentration of $\sim 29 \text{ mg}\cdot\text{L}^{-1}$. The resultant PLL-NPs suspension was stored at 4 °C for further applications.

2.3 Synthesis of enzymatic photoluminescent glucose nanosensors (GOx-NPs)

GOx molecules were conjugated to PLL-NPs through GA-assisted crosslinking. Specifically, 2.4 mg GOx molecules were dissolved in 8 mL of as-prepared PLL-NPs solution. After thoroughly mixing, 14.4 μL of GA solution (diluted by 100 times with water) was added into the mixture, followed by shaking with table concentrator for 2 h at room temperature to complete the reaction. The solution was centrifugated at 16000 r/min for 30 min, and GOx-NPs were collected and subsequently redispersed into 8 mL water with a concentration of $\sim 232 \text{ mg}\cdot\text{L}^{-1}$. The resultant GOx-NPs suspension was stored at 4 °C for further application.

2.4 Characterization

TEM images were obtained with an electron microscope (type Hitachi H-800) at an acceleration voltage of 120 kV, using NPs aqueous dispersion placed on the TEM specimen support (cuprum). Zeta potentials and hydrodynamic sizes were determined by photon correlation spectroscopy and by dynamic light scattering (DLS), respectively, using a Zetasizer Nano instrument (Malvern Instruments; www.malvern.com). UV-visible absorption and steady-state photoluminescent spectra were recorded on a UV-3101PC spectrophotometer (Shimadzu) and an F-4600 fluorescence spectrophotometer (Hitachi), respectively. Time-resolved fluorescence (TRF) measurements were performed on a commercial plate reader (Victor $\times$ 4; from Perkin-Elmer).

2.5 Ratiometric photoluminescence measurements and sensor calibration

The fluorescence intensity-based calibration was carried out in a standard spectrophotometric cuvette, in which 500 μL of air-saturated NPs-GOx solution was placed and sealed by 500 μL of mineral oil. To the cuvette bottom below oil layer, 125 μL of air-saturated glucose solution (in phosphate buffer saline buffer) with different concentrations were injected, respectively, to reach a final concentration of 0, 2, 4, 6, 8, 10 and 12 mM. Immediately after the addition of glucose, fluorescence spectra were recorded every 0.5 min with a scanning speed of 1200 nm/min under a 393-nm excitation. Because the photoluminescence intensity ratio was time-evolved, 8 seconds was further added to the registration time of each intensity ratio which considered the scanning speed and spectral range (490 - 650 nm).

2.6 Time-resolved fluorescence measurements and sensor calibration

The lifetime-based calibration was performed in a black 96-well microplate. Normally, 8-12 wells were used for one measurement, each filled with 100 μL of GOx-NPs solution containing bovine serum albumin (BSA, 0.07 g/mL). The microplate was initially conditioned for 20 min to equilibrate with air and at constant temperature (37 $^{\circ}\text{C}$). Then 25 μL of air-saturated glucose solution (in phosphate buffer saline buffer) with different concentrations were quickly injected, respectively, to reach a final concentration of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 mM. After addition of 200 μL of mineral oil to prevent ingress of air, fluorescence signal was immediately measured on a commercial plate reader (Victor $\times$ 4; from Perkin-Elmer). Rapid lifetime determination of phosphorescent nanosensors was performed based on time-resolved fluorescence (TRF) mode. [27, 29] Specifically, two TRF intensities were recorded after delay times of 30 μs (t_1) and 70 μs (t_2), each with a gating time of 100 μs and using 340 nm excitation and 642 nm emission filters. Measured TRF intensity signals were converted into lifetime (τ) values according to the following equation: $\tau = (t_1 - t_2)/\ln(F_1/F_2)$, where F_1 and F_2 correspond to the integrated signals over 100 μs as obtained after delay times of t_1 and t_2 , respectively.

2.7 Human serum test

GOx-NPs were utilized to detect glucose concentration of human serum samples by means of lifetime-based approach. Before measurements, all samples (obtained from Zhongguancun Hospital, China) were diluted 3 times by phosphate buffer at pH 7.0. In a black 96-well microplate, 100 μL of GOx-NPs solution was placed inside each well. The microplate was initially conditioned for 20 min to equilibrate with air and at constant temperature (37 $^{\circ}\text{C}$). Then 25 μL of diluted serum sample was quickly injected into the well, followed by addition of 200 μL of mineral oil. After 2 minutes, phosphorescence signals were measured on a commercial plate reader according to the procedures of TRF method, and the intensity signals were converted into lifetime consequently. Finally the concentration of glucose in each serum sample was acquired according to calibration curve with 2 minutes determination time.

3. Results and discussion

3.1 Synthesis and characterization of photoluminescent glucose nanosensor

Oxygen nanosensors (PLL-NPs) were firstly prepared according to previously reported PLL-assisted reprecipitation-encapsulation method. [26] It was demonstrated that abundant surface amino groups were present on the surface of PLL-NPs. Taking advantage of the GA-assisted Schiff-base reaction, therefore, GOx molecules were facilely conjugated to oxygen nanosensors via the crosslinking between primary amino groups, and enzymatic glucose nanosensors were thus derived (denoted as GOx-NPs). The above fabrication process is schematically illustrated in Fig. 1a, wherein cross section of GOx-NPs is depicted. Basically, the oxygen probe PtTFPP and reference dye C6 are randomly distributed inside the particle core consisting of PS and DTS, and GOx molecules are extended outwards from PLL shell. The sensing capability of GOx-NPs arises from the cooperation of GOx with oxygen probe - the former specifically catalyzes the oxidation of glucose into gluconic acid and hydrogen peroxide with the expense of oxygen, whilst the latter reports the decrease of dissolved oxygen by means of phosphorescence increase and thereby detects glucose indirectly. Because of conjugation with GOx, the average hydrodynamic diameter of GOx-NPs is considerably enlarged (~ 150 nm) comparing that of PLL-NPs (~ 75 nm), which is consistent with the TEM results (Fig. 1

b and 1c). Accordingly, surface charge of GOx-NPs is also changed from 35.7 mV to -13.8 mV (inset in Fig. 1b and 1c), corresponding to the transition of particle surface from positively charged PLL to negatively charged GOx. It needs to point out that some homo-coupled by-products may be produced during the GA-assisted coupling reaction. For PLL-NPs, it is difficult for them to form NPs-NPs because of the strong electrostatic repulsion. This can be confirmed by the TEM pictures wherein no dumbbell-typed particle is observed. For GOx-molecules, it may yield GOx-GOx conjugates as they are well dissolved in aqueous solution; but even so, GOx-GOx will be removed from GOx-NPs through high speed centrifugation as described in experimental section.

Conjugation of GOx with PLL-NPs is further characterized by absorption spectra. As displayed in Fig. 2a, the 276-nm absorption band in GOx is attributed to amino acid residues, [30] and the 393-nm absorption in PLL-NPs is to PtTFPP. The two bands are both present in GOx-NPs, demonstrating that GOx molecules are successfully coupled to PLL-NPs. Since GOx-NPs are constructed from PLL-NPs, the two NPs should be similar in fluorescent properties. Fig. 2b shows emission spectra of PLL-NPs and GOx-NPs. It can be seen that the probe phosphorescence of PtTFPP in the two nanosensors, as is expected, both peaks at 650 nm, but the maximum of reference fluorescence of C6 shifts from ~500 nm (PLL-NPs) to ~530 nm (GOx-NPs). It is known that GOx has an intrinsic fluorescence ranged from 480 to 580 nm (peaking at 520 nm). [31] In addition, imines (Schiff base) resulted from the crosslinking of GOx with PLL also contribute a little to the broad-band fluorescence. [32] Therefore, the superimposition of the above two emissions and that of C6 can reasonably explain the red-shift of reference fluorescence.

It is known that imines are labile to hydrolysis. Chemical stability of the glucose nanosensors in phosphate buffer saline (PBS) was thus studied by DLS and spectral measurement respectively (see *supporting information*, Fig. S1). The results show that the hydrodynamic diameter of GOx-NPs is slightly increased after 60 hours' incubation, whilst the photoluminescent intensity ratio is constant except for a trivial blue-shift of the reference fluorescence. Obviously GOx-NPs are stable enough for glucose detection as the assay time is in the order of minutes. Although a few imine linkages do hydrolyze in GOx-NPs, which relax the bounded GOx molecules (i.e. enlarged particle size) and reduce the contribution of imine to reference fluorescence (i.e. blue-shifted green emission), the coupling between GOx and NPs might via many imines thanks to the abundant amine groups on particle surface, which greatly improve the stability of nanosensors.

3.2 Calibration of NPs-GOx in ratiometric photoluminescence intensity

Sensitivity of as-prepared photoluminescent glucose nanosensor was firstly tested by using of the ratiometric approach, i.e. simultaneously detecting two signals by a single excitation wavelength. In comparison with single-intensity based sensing that can not give quantitative information unless in cases where the precise concentration of the probes is known, the internal-calibration nature of ratiometric method yields more robust signals. Figure 3a displays typical responsive emission spectra of GOx-NPs exposed to glucose solution (8 mM). It is evident that the probe 650-nm emission increases gradually with the oxidation of glucose, but the reference 530-nm emission keeps rather constant, i.e. the designed photoluminescent nanosensors are sensitive to glucose. Time-evolved emission spectra of GOx-NPs were also measured in response to other concentrations of glucose (see *supporting information*, Fig. S2). By defining R as the ratio

of the emission intensity of probe (at 650 nm) to that of reference (at 530 nm), photoluminescence intensity ratios of GOx-NPs are obtained and plotted against time in Fig. 3b. It can be seen that the evolution of intensity ratio differs a lot according to glucose concentration. The higher the concentration, the shorter the response time for a signal (intensity ratio) change of 90 % (from ~2 to 4 minutes). This is different from previously reported phosphorescent nano-PEBBLE glucose sensors which have a constant response time. [24] The discrepancy is ascribed to different calibration methods: nano-PEBBLE sensors were calibrated under ambient atmosphere, while GOx-NPs were airproofed by an oil layer. In the latter case, the consumption rate of oxygen is in direct proportion to glucose concentration since the amount of dissolved oxygen is limited.

In terms of calibration plot, a series of calibration curves can be constructed from Fig. 3b with different determination time (*supporting information*, Fig. S3). Short determination time renders calibration plots with low sensitivity, and long time results in ones with narrow detection range. Fig. 4 displays a characteristic calibration plot with ~2.5 minutes determination time. The *Y*-axis is defined as R/R_0 , where R_0 and R denote the photoluminescence intensity ratio (I_{650}/I_{530}) of GOx-NPs without and with the presence of glucose at certain concentrations, respectively. The experimental data are well fitted by a Boltzmann fit according to Eq. 1,

$$\frac{R}{R_0} = A_2 + \frac{A_1 - A_2}{1 + \exp(x - x_0)/dx} \quad (1)$$

where A_1 , A_2 , x_0 and dx are empirical parameters. The variable x is related to different glucose concentrations. As can be seen, the dynamic range (range within which 95% of the total signal change occurs) of the glucose nanosensors is 2 - 10 mM. As for the limit of detection (LOD) determined by linear regression method, it differs substantially according to determination time. For example, LOD is 2.35 mM and 1.18 mM, respectively, for determination time of 1 and 4 minutes [*supporting information*, Fig. S4]. It needs to point out that the sensing particle core of GOx-NPs actually follows an approximate linear behavior against dissolved oxygen. [27] The sigmoidal procession against glucose indicates the enzymatic nature of proposed sensing mechanism. [33]

3.3 Calibration of NPs-GOx in time-resolved fluorescence (TRF)

Although ratiometric approach features internal calibration, photobleaching of PtTFPP in GOx-NPs virtually undermines the sensing accuracy. It is noticed that the intensity of 650-nm emission is decreased by ~14 % after 5 minutes' irradiation at 393 nm, while that of C6 is rarely changed (*supporting information*, Fig. S2). The as-obtained intensity ratio of I_{650}/I_{530} therefore is smaller than the real one, and concentration of glucose might be underestimated as a result. As another robust fluorescence-based detection technique, lifetime-based measurement is usually deemed as a reliable means in that it is free of fluctuation from excitation sources as well as from concentration of probes. In this work, the time-resolved fluorescence microplate reader is employed to investigate lifetime-based response of GOx-NPs to glucose. It is found that the aftereffect of photobleaching on GOx-NPs is rather trivial, and only a 2% decrease of lifetime is resulted after 8 minutes' measurement (*supporting information*, Fig. S5). It was reported that fluorescence lifetime of single molecule was severely shortened by photobleaching. The slight reduction of lifetime of GOx-NPs should be thanks to the large amount of PtTFPP molecules per particle, only a tiny fraction of which is photobleached.

Figure 5a displays lifetime-based response of GOx-NPs against different glucose concentrations. It can be seen that phosphorescence lifetime of GOx-NPs increases gradually with the presence of glucose, corresponding to the diminishment of dissolved oxygen. Similar to the case of photoluminescent intensity ratio, the evolution of lifetime as well as response time is dependent on glucose concentration. Taking advantage of lifetime data with certain determination time, various calibration plots can be established (*supporting information*, Fig.S6). But only calibration curves with determination time longer than 2 minutes are of practical application, considering the detection sensitivity and dynamic range. Figure 5b displays a characteristic calibration plot with 2 min determination time, which can be well fitted by a Boltzmann function described in Eq. 1. The dynamic range is around 1- 6 mM, which is narrower than that obtained from intensity-based calibration. Also, the lifetime-derived LOD varies with determination time. It is 0.07 mM and 0.2 mM for determination time of 2 and 4 minutes respectively (*supporting information*, Fig. S7). The value is much lower than that from intensity-based approach, indicating that TRF mode can provide more sensitive detection of glucose.

3.4 Analysis of human serum sample

As a demonstration of the sensing capability, GOx-NPs were tested glucose in human serum samples. The ratiometric photoluminescence approach was adopted firstly. Unfortunately, the serum samples have a strong autofluorescence that superposes with the reference emission of GOx-NPs (peaking at 513 nm), which considerably distorts the ratiometric signals (*supporting information*, Fig. S8). In comparison, TRF mode is much suitable for determination of glucose in serum samples, as the 30- μ s delay can effectively eliminate background fluorescence whose lifetime is usually in the order of nanoseconds. Table 1 shows the as-obtained result of three human serum samples, in which the calibration curve in Fig. 5b is employed to convert measured lifetime data into glucose concentration. The errors (between -10.3% and -2.9%) are acceptable considering the complicated bio-components in serum, which may be unspecifically absorbed onto the surface of GOx-NPs and thereby hamper the enzyme catalytic reaction. Therefore, further modification such as PEGylation is needed for GOx-NPs to work well in bio-environments, and related work is being carried out.

4. Conclusions

In summary, photoluminescent enzymatic glucose nanosensors, GOx-NPs, are prepared by coupling GOx with PLL-coated nanoparticles doped with the reference dye C6 and probe PtTFPP through GA-assisted Schiff-base reaction. The GOx molecules reside on particle surface to catalyze glucose with the expense of oxygen, which is reported by the sensing particle core. Because of nanosized dimension (150 nm) and enzymatic nature, GOx-NPs are very sensitive to glucose with a quick response time of a few minutes. Two robust approaches, ratiometric photoluminescence and time-resolved fluorescence, are adopted for glucose calibration, and various calibration curves are resulted according to different determination time. Comparatively, the former technique gives rise to wide dynamic range (e.g. 2-10 mM) and high LOD values (about 1-2 mM), while the latter produces narrow dynamic range (e.g. 1-6 mM) with low LOD values (about 0.1-0.2 mM). In addition, glucose in serum was detected with GOx-NPs by means of TRF mode and reliable results are derived. With further surface biomodification, the glucose nanosensors are promising for *in vivo* sensing of glucose.

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Biographies

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Sample	Experiment data (mM)	Standard data (mM)	Relative error (%)
1	4.08	4.55	-10.3
2	5.94	6.36	-6.6
3	5.67	5.82	-2.9

Hong-shang Peng received his PhD degree from Beijing Jiaotong University in 2007 and now is a professor of Beijing Jiaotong University. His current research interests include fluorescent nanosensors for oxygen and temperature, and fluorescent bioimaging.

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Table 1. Determination of glucose in human serum samples based on TRF mode

Figure 1. (a) Schematic representation of the synthesis of photoluminescent glucose nanosensor GOx-NPs. From stage 1 to stage 2, PLL-NPs and GOx-NPs are sequentially prepared. (b) Characterizations of PLL-NPs and (c) GOx-NPs. DLS and the insets of TEM image and zeta potential, for respective NPs, are provided.

Figure 2. (a) Absorption spectra of GOx, PLL-NPs and GOx-NPs. (b) Emission spectra of PLL-NPs and GOx-NPs. The excitation wavelength is at 393 nm.

Figure 3. (a) Time-evolved emission spectra of GOx-NPs in response to glucose (8 mM). The spectra are recorded after glucose addition with a time interval of 0.5 min sequentially (room temperature), and the excitation wavelength is at 393 nm. (b) Kinetic response of GOx-NPs to increasing concentration of glucose in intensity ratio (I_{650}/I_{530}). The intensity ratios start at the time of 8 second and are compensated for photobleaching through controlled experiment. The concentration of glucose ranges from 2 to 4, 6, 8, 10 and 12 mM.

Figure 4. Ratiometric photoluminescence intensity-based calibration plot for GOx-NPs. The data are obtained from Fig. 3B with determination time of 158 s (~ 2.5 minutes).

Figure 5. (a) Kinetic response of GOx-NPs to increasing concentrations of glucose in phosphorescence lifetime. As indicated by the arrow, concentration of glucose is increased in sequence from 0 to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 mM. (b) Lifetime-based calibration plot for the glucose nanosensor. The data are obtained from Fig. 4a with determination time of 2 minutes.