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RESEARCH PAPER

Utilizing hyaluronic acid as a versatile platform for fluorescence resonance energy transfer-based glucose sensing

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Abstract

Here, we utilized the ultrasonic emulsification technique to generate hyaluronic acid microspheres incorporating a fluorescence-based glucose biosensor. We synthesized a novel lanthanide ion luminophore based on Eu^{3+}. Eu sulfosuccinimidyl dextran (Eu-dextran) and Alexa Fluor 647 sulfosuccinimidyl-ConA (Alexa Fluor 647-ConA) were encapsulated in hyaluronic acid hydrogel to generate microspheres. Glucose sensing was carried out using a fluorescence resonance energy transfer (FRET)-based assay principle. A proportional fluorescence intensity increase was found within a 0.5–10-mM glucose concentration range. The glucose-sensing strategy showed an excellent tolerance for potential interferents. Meanwhile, the fluorescent signal of hyaluronic acid microspheres was very stable after testing for 72 h in glucose solution. Overall, hyaluronic acid microspheres encapsulating sensing biomolecules offer a stable and biocompatible biosensor for a variety of applications including cell culture systems, tissue engineering, detection of blood glucose, etc.

Keywords Fluorescence resonance energy transfer · Glucose · Hyaluronic acid · Microspheres

Introduction

Accurate monitoring of glucose in vitro and in vivo has attracted more and more attention. This could be first attributed to the fact that glucose is the most significant energy source in metabolism processes, and its concentration in blood is considered as an indicator to human health conditions. Second, blood glucose level is an important parameter in clinical medicine, as whether it is too high or too low apart from the normal concentration level, it causes harmful effect to human health [1]. Furthermore, glycolysis is the key metabolic pathways of cells, and the consumption of glucose partly reflects the growth and metabolism of cells [2, 3]. Therefore, monitoring accurately of the glucose levels in blood and culture medium is of great importance for human health and scientific research.

Generally, the conventional methods of detecting glucose levels are based on spectrophotometry [4], fluorometry [5], electrochemistry [6], and chemiluminescence [7]. Although these methods were proved to be effective in many situations, they exposed some defects difficult to overcome for the direct monitoring of glucose levels in whole blood or cell culture medium. For instance, the methods, based on spectrophotometry and electrochemistry, can be easily disturbed by blood color and contamination of the electrode by proteins in whole blood or culture medium [8]. The application of chemiluminescence-based method is limited by the analogue interference in complex samples. Although the method based on fluorometry can overcome those limitations to a certain degree, they commonly suffer from the autofluorescent background by UV-vis excitation light sources [9]. Therefore, it is highly desired to develop technologies that are simple, anti-interference, and low cost for the monitoring of glucose levels in whole blood or cell culture medium.

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One of the more advantageous approaches is based on competitive binding and fluorescence resonance energy transfer (FRET) to detect glucose with fluorescence, as they enable glucose monitoring of sensitive samples with minimal disturbance [10]. A well-established format is based on a natural glucose-binding protein (lectin) concanavalin A (ConA) that is labeled with a fluorophore [11, 12]. Many efforts have been undertaken on the construction and optimization of such FRET biosensors. For example, lanthanide ions, particularly europium (Eu^{3+}) and terbium (Tb^{3+}), were employed due to the high background fluorescence and short lifetime of conventional dyes [13–15]. However, the applications of lanthanide ions are still limited by their potential toxicity and instability after a long time incubation [16, 17].

Recently, the use of hydrogel-based polymers with encapsulated biological components has provided a promising platform for the development of new biotechnology applications such as chemical sensing, cell encapsulation, and drug delivery [18]. The use of such polymers has been reported to improve diffusion of small molecules, such as glucose, and enhance signal-to-noise ratio, biocompatibility, and stability of implanted sensors [19]. Especially, Ballerstadt et al. developed several fiber-coupled fluorescence affinity sensors, which used Sepharose or collagen as encapsulation polymer, and performed well in glucose monitoring in rats and pigs [20, 21]. However, the dyes they used are Alexa 647 and 750, which still suffered from high background fluorescence and short lifetime.

Thus, this paper aimed to construct a FRET-based glucose sensor with a new lanthanide ion luminophore-based Eu^{3+} developed in our lab. In addition, the authors used hyaluronic acid (HA) hydrogel microspheres to encapsulating Eu-dextran and Alexa Fluor 647-ConA bioconjugates, which was expected to possess excellent performance and improved stability. HA is a natural polysaccharide abundant in extracellular matrix and has stood out due to its nontoxic, great water retention ability and biocompatibility, which enables it to be used in vivo [22]. However, their application in glucose monitoring is still rare. Thus, the detection accuracy, anti-interference ability, and biocompatibility of the constructed sensor were individually verified.

Materials and methods

Chemicals

Human hepatocellular carcinoma (HepG2) cells (ATCC HB8065) were graciously provided by Dr. Lifang Jiang (Zhejiang University, Hangzhou, China). Alexa Fluor 647 was obtained from Invitrogen (Carlsbad, CA, USA). Amino-dextran (MW 670 kDa), ConA (MW 102 kDa), D-(t) glucose, and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich. Bis(sulfosuccinimidyl)suberate (BS3) was obtained from Heowns. Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Eng. Material Co. Ltd. (Hangzhou, China). Dulbecco's modified Eagle's medium (DMEM) basal medium was purchased from Gibco (Gaithersburg, USA). All the other chemicals were of analytical grade and used as received. Double-distilled water used in this work was purified with a Milli-Q purification system (Barnstead, USA) to a specific resistance of 18 MΩ/cm.

Synthesis and characterization of luminescent europium complexes

We designed and synthesized the luminescent europium complexes (Fig. 1) in a previous work by referring to the relevant literature [23]. The europium complexes could be well dissolved in double-distilled water at room temperature, and the excitation/emission spectra of the europium complexes were tested using a fluorescence spectrophotometer (Shimadzu, RF-5301PC, Japan) in the aqueous solution state. The europium complex was dissolved in double-distilled water to 10 μg/mL as a test sample. In the process of the emission scan, we set the step size at 1 nm and the interval time at 500 ms for each step. The sample was excited at 334 nm to scan the fluorescence emission spectrum.

The luminescent quantum yield was determined by a relative method by using rhodamine 6G as a standard [24]. The lifetime measurements for the europium complexes were performed with EnSpire multimode microplate reader (PerkinElmer, USA), and the results indicated a lifetime of 1 ms with the fitting curve (see Electronic supplementary material (ESM) Fig. S1).

Labeling of fluorescent probe

To label the amino-dextran with luminescent Eu complexes, a tenfold molar excess of Eu was added to the amino-dextran

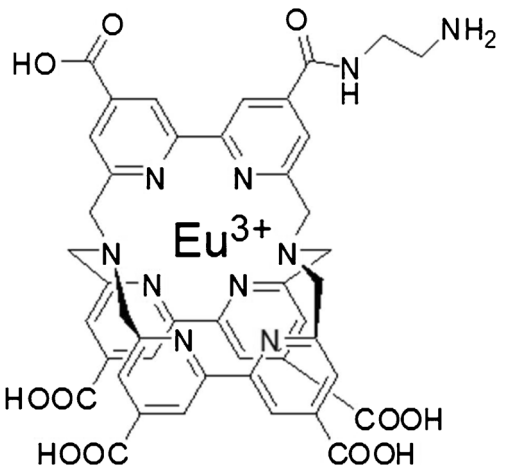


Fig. 1 The structure of the europium complexes

(6.67 μM) together with 0.5 mM of BS3 in phosphate-buffered saline (PBS) pH 7.4 containing 100 μM $CaCl_2$ and 100 μM $MnCl_2$ in a 100-μL reaction. The reaction proceeds 1 h at 25 °C. For the subsequent labeling of ConA with Alexa Fluor 647, a tenfold molar excess of Alexa Fluor 647 was added to 16.67 μM protein in PBS pH 7.4 containing 100 μM $CaCl_2$ and 100 μM $MnCl_2$, incubated 1 h at 25 °C. The labeled dextran/ConA was purified on a G-25 gel filtration column to remove any unconjugated fluorescent probe. Therefore, a pure sample of dextran/ConA-labeled fluorophore was obtained. After exciting at 334 nm, the fluorescence emission at 620 nm for the Eu conjugate and 661 nm for the Alexa Fluor647 conjugate was measured, respectively, using a fluorescence spectrophotometer (Hitachi, 650-10S, Japan). And the results indicated that the labeling efficiency was determined by calculating the concentration ratio of dextran/ConA and fluorophore. The anthrone-sulfuric acid colorimetry method was used to measure the concentration of dextran [25]. And the concentrations of ConA were determined by ultraviolet spectrophotometry [26]. The concentration of Eu and Alexa Fluor 647 was detected using an EnSpire multimode microplate reader (PerkinElmer, USA) at an excitation/emission wavelength of 334/620 and 650/670 nm and detected according to their respective calibration curves.

Preparation of HA microspheres

The HA microspheres were fabricated by the ultrasonic emulsification technique [27]. Briefly, the Alexa Fluor 647-ConA and Eu-dextran solutions were mixed in a ratio of 40:1 by mass in PBS. The mixture was stirred at 700 rpm for 1 h and added HA-CHO to 10 mg/mL. The liquid paraffin as dispersed phase was slowly incorporated into the HA-CHO mixture suspension with 1 wt% surfactant (Span-80:Tween-80 = 88:12, *v*/*v*) by mechanical stirring for 5 min using a mix-plus. A pre-emulsion was obtained and sequentially submitted to the ultrasonication process. The HA-ADH was dissolved in PBS to 10 mg/mL. The pre-emulsion was sonicated using a 5-mm diameter, 19-kHz ultrasonic probe (Unique, Disruptor, 800 W, Indaiatuba, Brazil) for 15 min with a slow addition of sodium HA-ADH. Afterwards, we obtained hyaluronic acid microspheres by centrifugation (7000 rpm for 15 min). The hyaluronic acid microspheres were washed twice with isopropyl alcohol to remove excessive liquid paraffin. The preparation of HA-CHO and HA-ADH was conducted as described in the ESM.

Once generated, the microspheres were transferred to PBS, followed by centrifugation (250 rpm for 3 min) and resuspension (3 times). Low centrifuge speed was used to ensure that capsules are not damaged during centrifugation. The microspheres were pipetted on a clean silicon wafer and dried for scanning electron microscopy (SEM) imaging.

Cytotoxicity test

HepG2 cell was cultured in DMEM medium with 10% FBS, 1% streptomycin, and penicillin antibiotics [28]. Cells were washed with PBS when it was reaching 80% of confluence, then treated with HA microspheres or incubated with Alexa Fluor 647-ConA and Eu-dextran that was blended into the medium in advance. Then, the cell culture plates were incubated at 37 °C for the time intervals indicated. For the control group, cells of the same number were seeded but no microspheres were added; 500 μL PBS was first added to each well to wash off the unattached dead cells. Then, to each well was added 500 μL PBS and 150 μL MTT solution (5 mg/mL dissolved in PBS) and incubated for 3 h at 37 °C. At the end of incubation, the solution was removed and the formazan crystals were dissolved in 300 μL DMSO for 1 h at 25 °C. Then, we used a Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT, USA) to measure the absorbance at a wavelength of 570 nm. Cell viability was expressed as the percentage of absorbance normalized to the negative control at the same time [29]. The control group was regarded as 100% cell viability. A blank control with only cell culture medium in the well was regarded as 0% cell viability.

Glucose assay protocols

Before encapsulated into HA hydrogel microspheres, the Eu-dextran and Alexa Fluor 647-ConA were collocated to determine the concentration of glucose in a millimolar range (as found in plasma) [30]. Each well of the 384-well assay plates (Corning 384-Well Multiwell Plates, USA) was filled with 5 μL of the Eu-dextran solution and 5 μL of the Alexa Fluor 647-ConA solution. The samples (10 μL) containing glucose in a concentration between 0.5 and 10 mM were dissolved in PBS (pH 7.4) containing 100 μM $CaCl_2$ and 100 μM $MnCl_2$. Then, we added the samples to all the wells, respectively, and mixed it evenly. After 45 min, the TRF function of the Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA) was used to detect the fluorescence signals of the 384-well assay plates. The sample was excited by 334 nm and the emission intensity was obtained from the peak value at 620 nm. And the delay time was set to 500 μs to achieve the time-resolved fluorescence signals. Control experiments were conducted by adding PBS buffer in place of glucose samples to give a blank signal. The blank was subtracted from the signal of fluorescent response to samples. All experiments were done in triplicates.

For glucose sensing, the HA microspheres, encapsulating Eu-dextran and Alexa Fluor 647-ConA, were incubated with 20 μL of different glucose concentrations for 45 min and pipetted on 384-well plates, respectively. Results were obtained by Varioskan™ LUX multimode microplate reader. Precautions were taken to keep the

experimental conditions constant compared with previous experiments (e.g., incubating time and temperature). To prove that the established glucose sensor can work in complicated biological matrixes, possible interferents were added into the glucose solution, and their potential interference in glucose sensing was kinetically analyzed by time-resolved fluorescence for HA microspheres, which meant a delay time of 500 μs was set. The HA microspheres were added into the glucose solution with a concentration between 0.5 and 10 mM containing 10% FBS. Then, the fluorescent signal of HA microspheres was read over 45 min between intervals of shaking. For the control experiments, PBS was added instead of FBS to give a blank signal. The blank was subtracted from the signal of fluorescent response to samples. All experiments were done in triplicate. Furthermore, 4 mg/mL for transferrin (Tf) and 55 mg/mL for albumin from bovine serum (BSA) were tested by adding them, respectively, into glucose solutions with the same method.

Validation of the glucose sensor using human blood sample

To further determine the feasibility of our glucose sensor in real biological samples, five tubes of human blood samples collected from healthy volunteers were detected. The samples of human serum were mixed with HA microspheres in microcentrifuge tubes. After incubating for 30 min, the incubation was transferred to the 384-well assay plates with 20 μL each well and detected by a Varioskan™ LUX multimode microplate reader. Finally, the results were compared with those obtained by Glucose Hexokinase FS (Diagnostic Systems GmbH, shanghai, China), which was considered to be a standard clinical method [31].

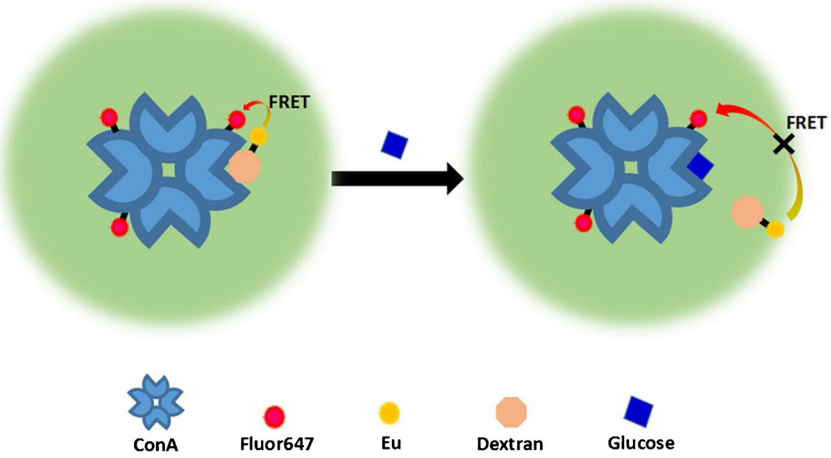
Statistical analysis

All data reported in the text are mean ± SD of three independent experiments each conducted in triplicates ($n = 9$). Evaluation of the data was carried out using the Statistical Package for Social Sciences (version 15.0, SPSS Inc., Chicago, IL, USA). Comparisons between multiple groups were performed with one-way ANOVA post hoc tests; results for different treatments were compared with the control groups using Dunnett's t test for statistical comparisons. A p value < 0.05 was determined to be significant.

Results and discussion

Sensing mechanism

Scheme 1 outlines the principle of the sensing mechanism for glucose detection. A Eu-dextran and Alexa Fluor 647-ConA biomolecular system offers the potential to monitor glucose levels by relying on FRET-based quenching mechanism. Briefly, when Alexa Fluor 647-ConA (acceptor) is introduced in a solution of Eu-dextran (donor), Alexa Fluor 647-ConA reversibly attaches to Eu-dextran resulting in a sufficiently small distance (< 10 nm). The small molecular proximity leads to quenching of the fluorescence signal of Eu-dextran. The addition of glucose to this system results in competitive binding of glucose to ConA and releases Eu-dextran from Alexa Fluor 647-ConA. Once released, Eu-dextran starts to fluoresce again, thereby creating a homogeneous glucose assay. Furthermore, based on the current results, the Förster critical distance of Eu-dextran and Alexa Fluor 647-ConA was calculated to be 7.36 nm (see ESM), indicating the detection was based on FRET methods [32].



Scheme 1 Schematic illustration of the principle of the glucose biosensor based on fluorescence resonance energy transfer

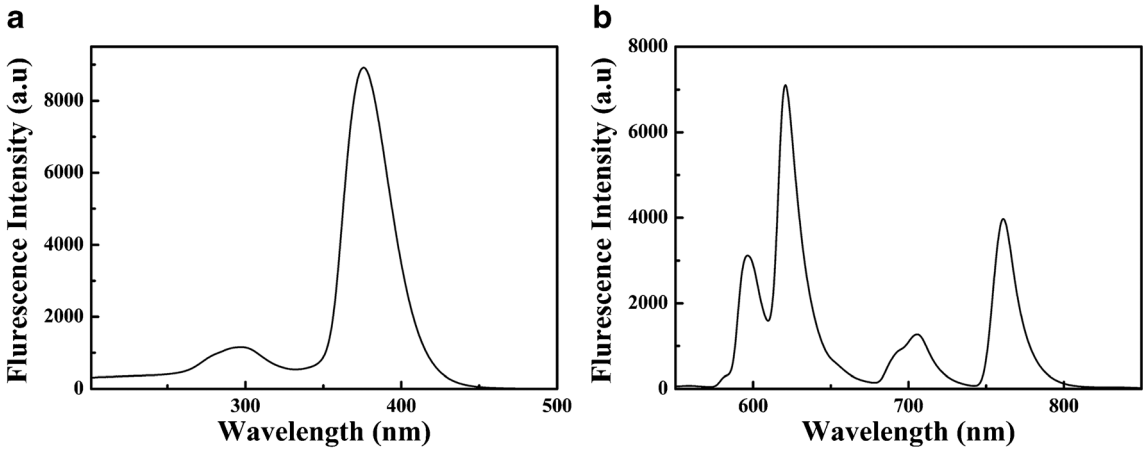


Fig. 2 Emission (**a**) and excitation spectra (**b**) of the europium complexes

Luminescent properties of the europium complexes

The quantum yield of the europium complexes was measured in diluted water solution at 30.34% (ESM Table S1) and that is quite excellent compared with similar europium complexes [33]. The fluorescence emission spectrum of the europium complexes (Fig. 2a) was obtained by excitation at 340 nm. There were several emission peaks at 585, 604, 620, 694, and 702 nm, which was the typical fluorescence emission spectrum for the lanthanide europium. According to the fluorescence emission spectrum, the intensity of emission fluorescence was the lowest at 630–670 nm, which provided the basis for their application in FRET. The fluorescence excitation spectra (Fig. 2b) showed that the europium complexes had the absorption peak at 340 nm. Excited by the absorption band at 340 nm, the Stoke shift reached 280 nm for the emission peak at 620 nm. Meanwhile, no emission was found for Alexa Fluor 647 (ESM Fig. S2) at 620 nm, indicating minor disturbance. Therefore, the combination of the europium complexes and Alexa Fluor 647 exhibited excellent fluorescence characteristics. Furthermore, the lifetime of the europium complexes was estimated to be 1 ms with the fitting curve (ESM Fig. S1). Therefore, the delay time was set to 500 μs when detecting the time-resolved fluorescence of the glucose sensor.

Characterization of HA microspheres

HA microspheres encapsulating Eu-dextran and Alexa Fluor 647-ConA were observed via bright field and fluorescence microscopy. The HA microsphere had an approximate size of 60 μm (Fig. 3a) and emitted bright fluorescence with Cy5.5 filter. Figure 3b showed that Eu-dextran and Alexa Fluor 647-ConA were steadily encapsulated into HA microspheres despite several washes. The distribution of Eu-dextran and Alexa Fluor 647-ConA was uniform within the HA microspheres. In addition, the approximate size of the HA microspheres was found to be about 35 μm due to dehydration (Fig. 3c). The surface of the microspheres was found to consist of multiple folds and appeared wrinkled.

HA microspheres for glucose sensing

We take the relative value of rising fluorescence intensity as the reference value to determine the glucose concentration. Figure 4 showed a normalized Eu-dextran and Alexa Fluor 647-ConA fluorescence intensity in response to different glucose concentrations. The calibration curve was obtained from 0.5 to 10 mM

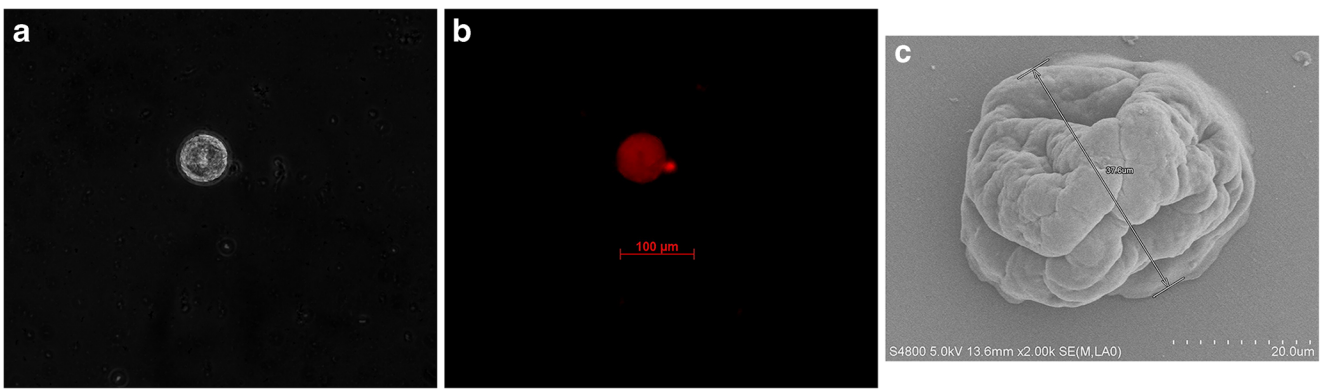


Fig. 3 **a** Bright-field image of microspheres with approximate size of 60 mm. **b** Observation of HA microspheres through Cy5.5 filter (Ex. 675 nm and Em. 694 nm). **c** SEM image of HA microspheres with approximate size of 35 μm

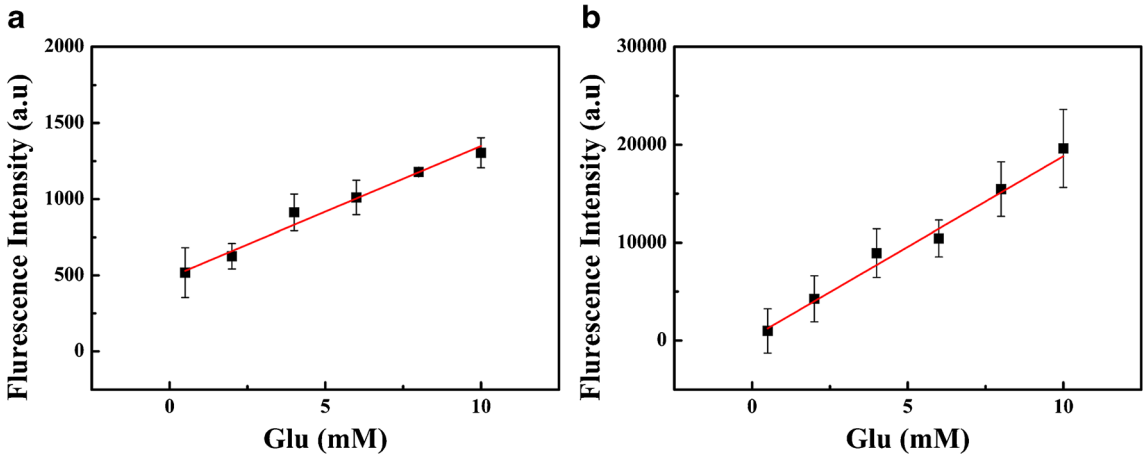


Fig. 4 Fluorescence intensity response to glucose, whose concentrations varied from 0.5 to 10 mM, with (**a**) and without encapsulating (**b**)

glucose concentrations, showing a concentration-proportional response. Such a linear dynamic range is wide enough to monitor the blood and cell culture medium glucose level [30]. Comparing the results before and after encapsulating Eu-dextran and Alexa Fluor 647-ConA into HA microspheres, no difference was found in the sensitivity of glucose sensing. Readjusting reagent ratios of Eu-dextran and Alexa Fluor 647-ConA in HA microspheres can result in faster response and increased linearity range [34].

The anti-interference capability of HA microspheres in blood samples and cell culture medium was tested by adding individual potential interferents (Tf, BSA, and FBS) into the glucose solution. The amount of each potential interferent added was equal to or higher than that in human blood or cell culture medium [35]. Figure 5 shows that the potential interferents did not bring any significant effects on the glucose assay and a linear calibration curve for glucose in the range of 0.5–10 mM was obtained, respectively. Such phenomenon might be contributed for two reasons: first, lanthanide complexes usually had a long decay time, typically between 0.01 and 10 ms, which could effectively eliminate background such as the intrinsic fluorescence of proteins (nanosecond); second, the hydrogel networks kept the macromolecules in the samples out of the HA microspheres.

Determination of glucose in human blood sample

Initially, the concentrations of glucose for the samples were measured using HA microspheres with 6.35, 5.35, 7.50, 6.34, and 6.90 mmol/L, while the corresponding results obtained by hexokinase methods were 4.36, 3.89, 4.71, 4.28, and 4.50 mmol/L. After plotting the values obtained by HA microspheres versus that detected by hexokinase methods (Fig. 6), a high linearity ($R^2 = 0.98$) was found. It seemed that the glucose sensor was sufficient for the preliminary estimate of glucose for real biological samples.

Stability tests for fluorescence of HA microspheres

To determine the stability of fluorescence, HA microspheres encapsulating Eu-dextran and Alexa Fluor 647-ConA were incubated with 5 mM glucose solution at 37 °C. We monitored the fluorescent responses of HA microspheres at regular intervals. As shown in Fig. 7, the fluorescent signal of HA microspheres did not change significantly, indicating a high stability during 72 h preservation. Such phenomenon could be first attributed to the fact that the europium complexes were chelates produced by the lanthanide europium and

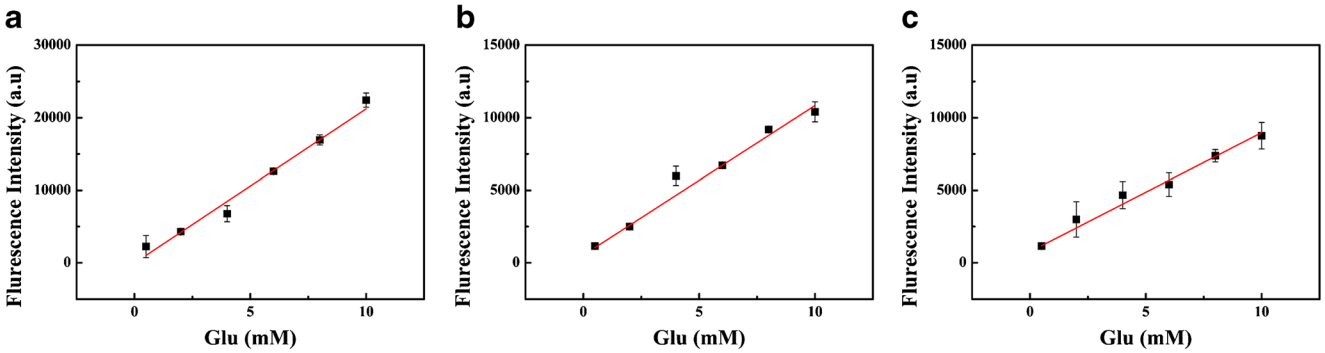


Fig. 5 Effects of various potential interferents on fluorescence responses of HA microspheres. **a** 4 mg/mL Tf. **b** 55 mg/mL BSA. **c** Tenfold-diluted FBS

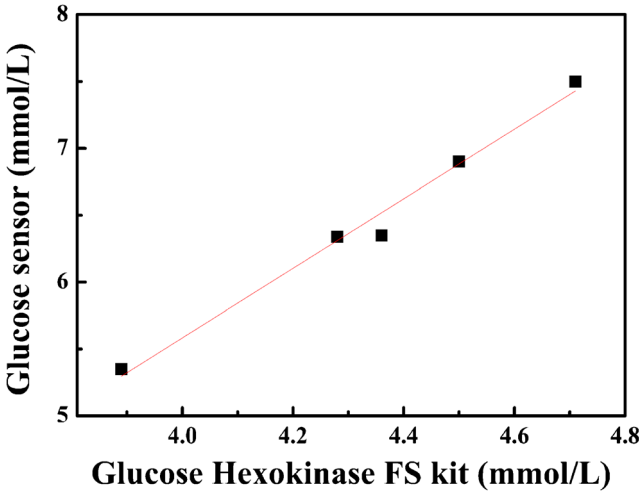


Fig. 6 Plots of detected glucose concentration from glucose sensor versus that from Glucose Hexokinase FS kits

EDTA derivatives, which enhanced the stability of fluorescence significantly [16]. Another reason could be that the hydrogel networks blocked ConA from irreversible aggregation [34].

Cytotoxicity of HA microspheres

As shown in Fig. 8, the HA microspheres encapsulating Eu-dextran and Alexa Fluor 647-ConA showed a favorable safety. For the glucose detection system, the major contributor to cytotoxicity was the lanthanide element [36]. The cell viability decreased significantly with the increase of Eu concentration. For example, the incubation process containing 200 nM Eu without being encapsulated into HA hydrogel microspheres induced significant death (about 40%) for HepG2. But the mesh structure of the HA hydrogel could insulate the reaction system from the external environment, thus reducing the toxicity of lanthanide.

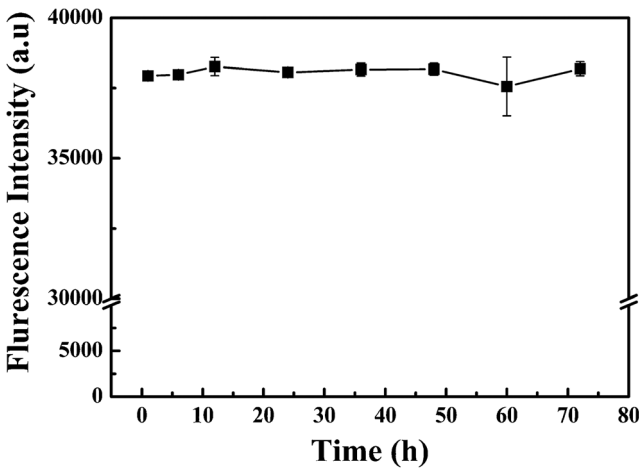


Fig. 7 Responses of HA microspheres encapsulating Eu-dextran and Alexa Fluor 647-ConA to 5 mM glucose sample within 72 h

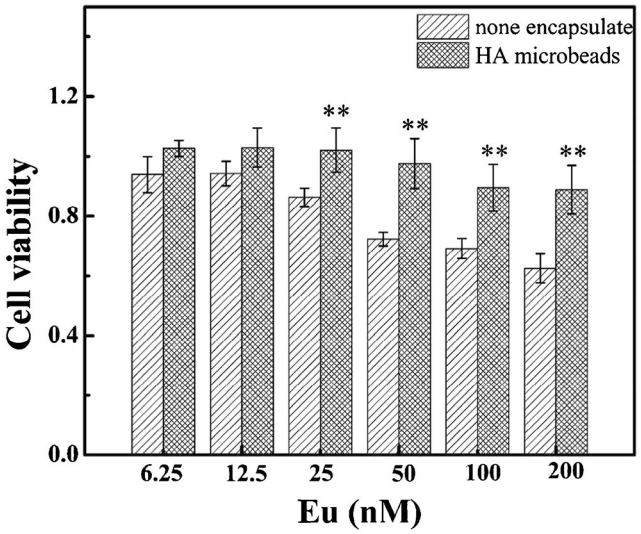


Fig. 8 Toxicity of HA microspheres in HepG2 cells. The concentration of Eu was used as a reference for cytotoxicity. The control group was regarded as 100% cell viability. A p value < 0.05 was determined to be significant according to hypothesis testing

Conclusion

Herein, we developed a HA-based glucose sensor with luminophore-based Eu^{3+}. The HA microspheres had an approximate size of 60 μm in aqueous environments and exhibited a proportional fluorescence intensity response at the concentration between 0.5 and 10 mM. More importantly, the glucose assay has additional features including excellent biocompatibility, stability of fluorescence, and good sensitivity. Still, further improvements in probe design and encapsulating hydrogel will serve to make lanthanide-based sensing a routine tool for biological detections.

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Compliance with ethical standards

Written informed consent was obtained from all individual participants and blood samples for publication of this study. The experimental procedures, sample processing methods, and possible consequences are informed in advance. The contents of the informed consent are strictly enforced.

The author(s) promised that the study was performed according to the international, national, and institutional rules considering animal experiments, clinical studies, and biodiversity rights. The study was approved by the Ethics Committee of Suzhou Institute of Biomedical and Technology, Chinese Academy of Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

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