Injectable hydrogel microbeads for fluorescence-based in vivo continuous glucose monitoring

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Edited by Mark E. Davis, California Institute of Technology, Pasadena, CA, and approved August 17, 2010 (received for review May 20, 2010)

Fluorescent microbeads hold great promise for in vivo continuous glucose monitoring with wireless transdermal transmission and long-lasting activity. The full potential of fluorescent microbeads has yet to be realized due to insufficient intensity for transdermal transmission and material toxicity. This paper illustrates the highly-sensitive, biostable, long-lasting, and injectable fluorescent microbeads for in vivo continuous glucose monitoring. We synthesized a fluorescent monomer composed of glucose-recognition sites, a fluorogenic site, spacers, and polymerization sites. The spacers are designed to be long and hydrophilic for increasing opportunities to bind glucose molecules; consequently, the fluorescent monomers enable high-intensity responsiveness to glucose. We then fabricated injectable-sized fluorescent polyacrylamide hydrogel beads with high uniformity and high throughput. We found that our fluorescent beads provide sufficient intensity to transdermally monitor glucose concentrations in vivo. The fluorescence intensity successfully traced the blood glucose concentration fluctuation, indicating our method has potential uses in highly-sensitive and minimally invasive continuous blood glucose monitoring.

Diabetes is a global pandemic affecting over 200 million people (1, 2). Maintaining normal blood glucose concentrations is crucial for preventing diabetic complications in the heart, kidney, retina, and neural system (3–6). The fingertip prick method for collecting a blood sample is used at present to accurately analyze blood glucose concentrations. However, the method provides intermittent information concerning glucose concentrations, which is not suitable to predict the trend of blood glucose change. In contrast, continuous glucose monitoring (CGM) allows diabetic patients to effortlessly recognize changes in blood glucose concentrations and signals a warning in the case of hyperglycemia patients; even when diabetic patients are sleeping (7, 8).

Fully-implantable glucose sensors, embedded in the body, are ideal for CGM. Previously, microdialysis and enzyme-tipped catheters have been developed as fully-implantable glucose sensors for CGM. Although these sensors are capable of providing the sequential information of blood glucose concentrations to diabetic patients, they need to have external links for continuously collecting samples or transmitting signals; thereby these methods cause discomfort and the risk of infection. Recently, an optical method using fluorescent beads (9–12) was proposed for CGM. This method provides wireless transmission through the skin, and long-lasting activity in vivo compared to enzyme-based methods (13–16) that require an electrochemical reaction. However, fully-implantable glucose sensors based on the fluorescent principle have not yet been developed mainly due to the insufficient fluorescent intensity required for transdermal detection and the toxicity of the material (17).

Here, we developed the highly-sensitive, biostable, long-lasting, and injectable fluorescent microbeads for in vivo CGM (Fig. 1) to solve the aforementioned problems. We designed and synthesized a unique fluorescent monomer based on diboronic acid that enables reversible responsiveness to glucose without any reagents and enzymes. The fluorescent monomer has long, hydrophilic spacers and polymerization sites to bind flexible supports. As a result, the fluorescent monomer shows high mobility originating from the increase in opportunities to contact glucose molecules. Therefore, the fluorescent monomer has sufficient intensity for in vivo transdermal monitoring; even when it is immobilized in a solid support. Then, we immobilized the fluorescent monomer in microbeads. Due to the virtue of their small size, the fluorescent microbeads are injectable, minimally invasive, and rapidly respond to glucose change. By employing microfluidic devices, we succeeded in fabricating the microbeads of the order of $1 \times 10^6 \mu m$ with high throughput and high uniformity. Then, we experimentally verified in vitro and in vivo glucose monitoring. This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1006911107/-/DCSupplemental.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1006911107/-/DCSupplemental.

17894–17898 | PNAS | October 19, 2010 | vol. 107 | no. 42

www.pnas.org/cgi/doi/10.1073/pnas.1006911107
responsiveness of the fluorescent microbeads to show the potential of applying to in vivo CGM.

Results and Discussion

Design and Synthesis of Glucose-Responsive Monomer. Our glucose-responsive fluorescent monomer (GF-monomer) was modified from previously developed glucose-responsive fluorescent dye (GF-dye) (18). The glucose sensing principle of GF-dye is briefly explained as follows: GF-dye comprises of diboronic acid moiety and an anthracene moiety that act as the specific glucose-recognition site and the fluorogenic site, respectively. In the absence of glucose molecules, the fluorescence of the anthracene is quenched by a photo-induced electron transfer (PET) that occurs from the unshared electron pair of the nitrogen atom to the anthracene. When glucose molecules bind to the diboronic acid, a strong reaction between the nitrogen atom and boron atom inhibits PET. As a result, the fluorescence of anthracene becomes higher (Fig. 2A) compared to glucose-free condition (18–21). In addition, the diboronic acid moiety has a high selectivity of glucose due to the more than 10 times stronger reaction between the glucose-recognition site and the glucose compared to other sugars (22).

To apply fully-implantable glucose sensors, GF-dye needs to be immobilized to a solid support. However, the fluorescence intensity of GF-dye decreased 14 times after immobilization due to the decrease in the mobility of GF-dye (18); the decrease in mobility induced the decrease in opportunities to bind glucose molecules. Here, we synthesized GF-monomer from GF-dye to have better mobility even when GF-monomer is immobilized. There are two design challenges: a long spacer and a polymerization site that binds to a sparse and flexible immobilization material. We employed polyethylene glycol (PEG) and acrylamide (AAm) group as the spacers and the polymerization site, respectively. Advantages of using PEG was that PEG is not only long (23) (Mw = 3,400) but also hydrophilic (24). Thus, the PEG spacer allows better contact between the GF-monomer and water-soluble glucose molecules. Meanwhile, AAm group facilitates the polymerization of polyacrylamide (PAAm) gel that is a malleable, flexible hydrogel, with excellent water-binding capabilities (hydrophilicity) and biocompatibility (25). The sparse matrix and hydrophilic characteristics of PAAm gel contribute to the highly sensitive glucose responsiveness of GF-monomer even when immobilized in hydrogel scaffolds. Moreover, the biocompatibility of PAAm gel makes it possible to carry out in vivo application.

As described in Materials and Methods, GF-monomer was synthesized in a way that met the aforementioned requirements. (Fig. 2B). First, PEG with acrylamide group and carboxyl group (AAm-PEG-COOH) was prepared by coupling amino-PEG-carboxyl acid (NH₂-PEG-COOH) and acrylyl chloride. Then, the double amino terminated the fluorescent dye (GF-dye) and the prepared AAm-PEG-COOH were coupled in the presence of dehydration-condensation agent to obtain GF-monomer. Glucose-responsive fluorescent hydrogel (GF-hydrogel) (Fig. 2C) was obtained by polymerization of GF-monomer with AAm monomers and crosslinkers (see SI Text and Fig. S1).

Fig. 2. Glucose sensing principle and synthetic scheme of the fluorescent monomer (GF-monomer) and hydrogel (GF-hydrogel). (A) Fluorescence intensity changes depending on the existence of a glucose molecule. (B) GF-monomer is obtained by coupling the diboronic dye (GF-dye) and long, hydrophilic spacer with polymerization group (AAm-PEG-COOH). (C) GF-monomer is immobilized in polyacrylamide hydrogels having a sparse and flexible matrix.
Fabrication of the Fluorescent Microbeads. We fabricated glucose-responsive fluorescent microbeads (GF-beads), using a axisymmetric flow-focusing microfluidics device (AFFD) (26–28) that can produce monodisperse droplets with high uniformity (Fig. 3A). After gelation of the obtained droplets, we washed GF-beads to remove remaining unreacted GF-monomer. GF-beads were stored in buffer solution (Fig. 3B) with Pluronics® surfactant to prevent the adhesion of GF-beads to the surface of vials, tips, and needles; this surface treatment results in easy handling of GF-beads during implantation. The fluorescent image of GF-beads (Fig. 3C) reveals that GF-monomer was successfully immobilized in the PAAm hydrogel microbeads. The diameter of GF-beads was measured as 130 μm with narrow size distribution (see SI Text and Fig. S2). The size of GF-beads was smaller than the inner diameter of a general injection needle. The injectable size of GF-beads can minimize the damage to tissues when the microbeads are implanted in vivo. Furthermore, the beads of 130μm in diameter, unlike smaller sized beads (<1 μm), can stay between tissue layers without permeating the cell-membrane.

In Vitro Glucose Responsiveness Test. We performed the in vitro glucose responsiveness test using the fabricated GF-beads (see SI Text). From the fluorescent images for various glucose concentrations (Fig. 4 A–C), the glucose response curves were obtained for the glucose concentration of 0–1,000 mg·dL⁻¹ at the emission wavelength of 488 nanometers (nm) (see Fig. S3). When the glucose concentration increased from 0 mg·dL⁻¹ to 1,000 mg·dL⁻¹, the fluorescence intensity also increased depending on the glucose concentration (see Table S1). When the glucose concentra-

tion decreased from 1,000 mg·dL⁻¹ to 0 mg·dL⁻¹, the fluorescence intensity concurrently decreased depending on the glucose concentration (see Table S1). The fluorescence intensity curve for the increase in glucose concentrations matched the fluorescence intensity curve for the decrease in glucose concentrations (Fig. 4D). These results indicate that glucose association and dissociation occur reversibly in the glucose-recognition site of GF-monomer. The fluorescence intensity of GF-beads provided a suitable curve for measuring blood glucose concentrations in the physiological range (62.5–500 mg·dL⁻¹). At a glucose concentration of 500 mg·dL⁻¹, the relative fluorescence intensity of GF-beads was approximately 3 times higher than that of GF-dye immobilized in a rigid membrane (18).

We also measured the influence of photobleaching in our system, as shown in Fig. S4. As a result, the fluorescence intensity of the microbeads was reduced to 80% after 25 min when we continuously applied the excitation light (405 nm, 5.7 mW·cm⁻²) to the fluorescent microbeads. To minimize photobleaching of the microbeads, we believe that a pulse excitation system can be applied to long-term CGM. For example, if the fluorescence intensity of the microbeads is measured every 5 min with 1 ms expose, the photobleaching of microbeads will be less than 1% after 3 months.

In Vivo Glucose Monitoring. We implanted GF-beads into the ear skin of mice to test the hypothesis that the fluorescence intensity of GF-beads is transdermally detectable (see SI Text). We selected the ear skin for the implantation site due to its higher transmission (see Fig. S5A) compared to the other tissues such as abdominal muscle and urinary bladder (see Fig. S5B). GF-beads were implanted under the ear skin of a mouse (see Fig. S7) using an injection needle commonly used in a clinical setting. The implanted GF-beads, as shown in Fig. 5 A and B, were visible even though skin layers thicker than 200 μm with biological noises, such as tissues, hair, and secretions. In addition, mice possessing the implanted GF-beads in their ears moved as usual after implantation (see Movie S1), and remained alive without showing any abnormalities for over 30 days.

We also experimentally verified the correlation between the fluorescence intensity of the implanted GF-beads and the in vivo blood glucose concentrations by nine intravenous glucose challenges in five mice. Using the process as described in SI Text, we intravenously injected glucose to temporally elevate up to 370 mg·dL⁻¹ within the hyperglycemic range, and insulin to decrease to 130 mg·dL⁻¹ within the euglycemic range. Blood
glucose concentrations were measured with the glucose sensors by using a blood sample from the snipped tail. Fig. 5D and E illustrate the fluorescent images of the implantation site in the mouse ear (Fig. 5C) before and after the glucose challenge; pseudocolored images of Fig. 5D and E are shown in Fig. 5F and G, respectively. Fig. 5H plots the measured blood glucose concentrations and the fluorescence intensity of GF-beads by time. The fluorescence intensity constantly tracked the blood glucose concentration fluctuation of one or two up-and-down cycles, as shown in Fig. 5H and Fig. S6. The response of fluorescence intensity lagged 11 ± 5 min behind the change in blood glucose concentrations. Since the fluorescence intensity reflects the glucose concentration in subdermal interstitial tissues, the time lag mainly originated from the time lag of the changes in subdermal interstitial glucose concentration behind the changes in blood glucose concentrations (29, 30).

Conclusions
Our material has highly sensitive glucose responsiveness originating from the high mobility of GF-monomer. We combined microfluidics technology to immobilize our material in injectable-sized microbeads. These beads provide minimally invasive implantation and a fluorescent signal by transdermal transmission without any external links or electric power sources. The fluorescence intensity of the microbeads continuously corresponds to blood glucose concentrations in vivo, showing practical and efficient glucose monitoring. Due to the virtue of malleable PAAm gel, we can optimize the shapes of the glucose-responsive hydrogel depending on the implantation methods and sites. Moreover, since hydrogels are generally used in sensors to recognize specific molecules (31, 32) and controlled release carriers (33), the fully-implantable fluorescent microbeads provide a unique route to the intelligent, versatile CGM sensors.

Materials and Methods

Synthesis of Glucose-Responsive Monomer. Synthetic scheme of GF-monomer is described in Fig. 2B. The AAm-PEG-COOH (2.0 g) was dissolved in dichloromethane (12 mL) at 0 °C. Disopropylethylamine (DIEA) (460 μL) and acryloyl chloride (216 μL) were added to the solution and reacted for 60 min. The reactant solution was evaporated and applied to silica gel chromatography for purification. Appropriate fractions were dried under reduced pressure, then amino and carboxyl terminated PEG (NH₂-PEG-COOH) (1.40 g) was obtained. GF-dye (108 mg) was dissolved in ethanol (3 mL), NH₂-PEG-COOH (1,080 mg), Milli Q water (7 mL), DIEA (105 μL), and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (130 mg) were added to the solution under a nitrogen atmosphere. Next, the mixture was allowed to react at 25 °C for 3 h. The reactant solution was evaporated and dried under reduced pressure. Then, we obtained crude GF-monomer. The product was dissolved in a mixture of acetonitrile and Milli Q and then purified using the RP-HPLC. Appropriate fractions were evaporated and then lyophilized to obtain pure GF-monomer (235 mg). See SI Text for additional details.

Fabrication of Fluorescent Microbeads Using a Microfluidic System. The GF-beads were fabricated using an AFFD. We designed the AFFD with three-dimensional modelling software (Rhinoceros, AppliCraft) and used a stereolithography modelling machine (Perfactory, Envision Tec.) to fabricate the AFFD. The pregel solution contained 5 wt% GF-monomer, 10 wt% AAm, 0.2 wt% Bis-AAm, and 0.09 wt% sodium persulfate (SPS) in 60 mM phosphate buffer with 1.0 mM EDTA, pH 7.4. The pregel solution flows into the inner channel of the AFFD, while silicone oil (Dow Corning Toray Co., Ltd.) flows into its outer channel. The inlets of pregel solution and silicone oil were connected to a syringe pump (KDScientific) through ethylene tetrafluoroethylene tubes of 0.5 mm diameter. We set the flow rates of the inner and outer fluids at 10 μL·min⁻¹ and 150 μL·min⁻¹, respectively. The pregel solution was broken into droplets at the orifice by the flow of silicone oil. The collected droplets were gathered in phosphate buffer with N,N,N',N'-tetramethylethylenediamine at 37 °C. After 30 min, we washed the polymerized GF-beads three times with hexane (Kanto Chemical Co., Inc.), ethanol, Milli Q water, and phosphate buffer. In addition, Pluronic® P127 (Sigma-Aldrich) surfactant was dissolved in buffer solution (0.05 wt%) to prevent adhesion of the microbeads to the surface of vials, tips, and needles. See SI Text and Fig. S2 for additional details.

ACKNOWLEDGMENTS. We appreciate the support by Ms. N. Yamamoto for animal experiments. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).


