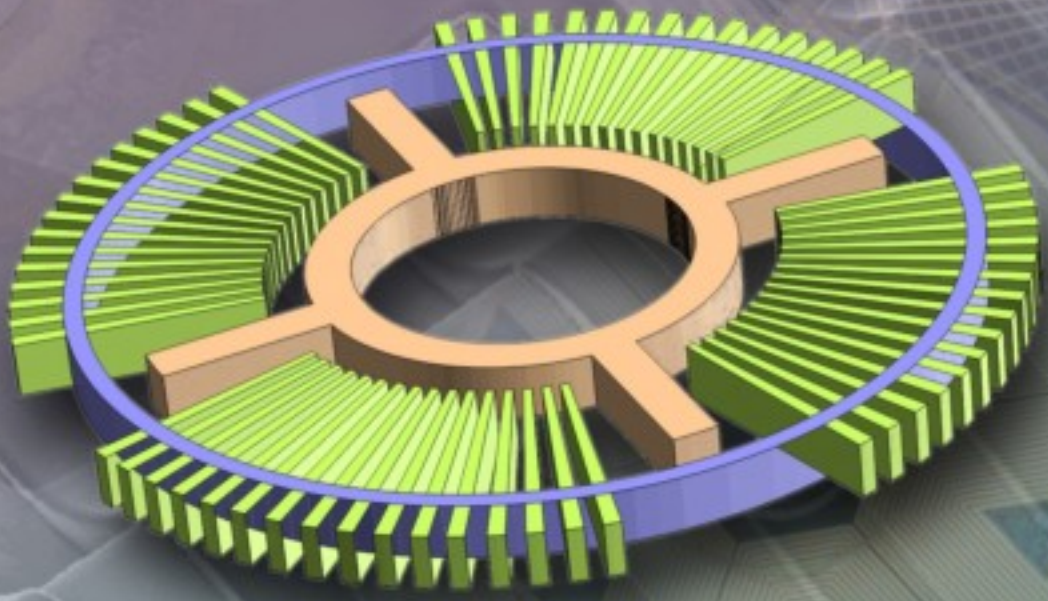
ISSN 1726-5749

SENSORS & TRANSDUCERS

12 vol. 3 Special /08



Microsystems: Technology and Applications

International Frequency Sensor Association Publishing

IFSA

Sensors & Transducers

Volume 3
Special Issue
December 2008

www.sensorsportal.com

ISSN 1726-5479

Sensors & Transducers Journal (ISSN 1726-5479) is a peer review international journal published monthly online by International Frequency Sensor Association (IFSA).
Available in electronic and CD-ROM.

Sensors & Transducers Journal

Contents

Volume 3
Special Issue
December 2008

www.sensorsportal.com

ISSN 1726-5479

Research Articles

Authors are encouraged to submit article in MS Word (doc) and Acrobat (pdf) formats by e-mail: editor@sensorsportal.com
Please visit journal's webpage with preparation instructions: http://www.sensorsportal.com/HTML/DIGEST/Submition.htm

Sensors & Transducers Journal, Vol. 3, Special Issue, December 2008, pp. 71-79

www.sensorsportal.com

Sensors & Transducers

ISSN 1726-5479

http://www.sensorsportal.com

NIR FRET Fluorophores for Use as an Implantable Glucose Biosensor

Majed DWEIK and Sheila A. GRANT
Department of Life and Physical Sciences - Corporative Research and Extension,
Lincoln University, 820 Chestnut Street Jefferson City, MO, USA
Tel.: 001-573-681-5134
E-mail: dweikm@lincolnu.edu

Received: 31 October 2008 /Accepted: 7 November 2008 /Published: 8 December 2008

Abstract: Development of an *in vivo* optical sensor requires the utilization of Near Infra Red (NIR) fluorophores due to their ability to operate within the biological tissue window. Alexa Fluor 750 (AF750) and Alexa Fluor 680 (AF680) were examined as potential NIR fluorophores for an *in vivo* fluorescence resonance energy transfer (FRET) glucose biosensor. AF680 and AF750 found to be a FRET pair and percent energy transfer was calculated. Next, the tested dye pair was utilized in a competitive binding assay in order to detect glucose. Concanavalin A (Con A) and dextran have binding affinity, but in the presence of glucose, glucose displaces dextran due to its higher affinity to Con A than dextran. Finally, the percent signal transfer through porcine skin was examined. The results showed with approximately 4.0 mm porcine skin thickness, 1.98 % of the fluorescence was transmitted and captured by the detector. *Copyright © 2008 IFSA.*

Keywords: FRET, NIR, Dextran, Concanavalin A, Glucose biosensor

1. Introduction

According to the American Diabetes Association (ADA) by the year 2020, 250 million people will be afflicted with diabetes [1]. Most diabetics monitor their glucose levels at least seven times a day, followed by appropriate actions necessary to maintain normal glycemia. Close monitoring of glucose level would help prevent some of the adverse health effects associated with diabetes, such as kidney failure, amputations, and blindness. An implantable glucose sensor would maintain stricter control over blood glucose levels while eliminating the need for blood drop testing.

Many researchers have pursued the development of sensor and biosensor devices for the long-term monitoring and managing of diabetes [2-5]. Medtronic/Mini-Med, has developed an electrochemical implantable glucose sensor. This sensor can operate for 72 hours subcutaneously. After 72 hours, the sensor becomes inoperable due to biofouling. Another problem with this sensor is the frequent re-calibration.

Other scientists are investigating implantable glucose sensors based on fluorescence spectroscopy due to the fact that fluorescence spectroscopy has the advantages of high sensitivity and superior specificity provided by molecular recognition [3]. For example, a series of experiments were performed using FRET (fluorescence resonance energy transfer) and a competitive binding technique. McShane et al. [4] utilized Concanavalin A and dextran which were labeled with acceptor (TRITC) and donor (FITC) dyes respectively. In the presence of glucose, the labeled dextran was displaced from the Concanavalin A. This resulted in a change in fluorescence. McShane achieved a detection range of 0–140 mg/dL glucose with a response time of less than 2 min [4]. The ratiometric nature of the FRET analysis method allowed compensation for variations in instrumental parameters, assay component concentrations, and measurement configuration [5]. However, as an implantable biosensor, the chosen fluorophores (TRITC and FITC) suffer from optical absorption due to hemoglobin; i.e., the wavelengths do not fall within the tissue window.

Fluorophores utilized as implantable sensors require high quantum yield and near IR emission (NIR), but such a combination does not currently exist because red and NIR dyes generally have lower quantum yields than the shorter-wavelength dyes. Table 1 lists properties of the ideal FRET fluorophore pair. A judicious choice of the FRET dyes is critical in the development of an optical implantable glucose sensor. In this study, it was hypothesized that the fluorophores Alexa Fluor 750 and Alexa Fluor 680 are viable choices for creating an implantable FRET biosensor capable of detecting glucose.

Table 1. Properties of an ideal fluorophore and pair.

1. High quantum yield
2. Non-toxicity
3. High tissue penetration
4. Large Stokes shift
5. Sharply spiked emission wavelengths
6. Long excited-state lifetime
7. Insensitivity to pH
8. Photo-stability when conjugated
9. Strong energy transfer

Alexa Fluor 750 (AF750) dye has a peak excitation at ~752 nm with its fluorescence emission maximum at ~779 nm. It is one of the longest wavelengths of Alexa Fluor dyes currently available with a spectrum similar to the commonly utilized Cy7 dye. Since it is well separated from frequently used far-red fluorophores such as Alexa Fluor 647, Alexa Fluor 660, or allophycocyanin (APC), the use of AF750 facilitates multicolor analysis. Additionally, conjugates of the AF750 dye become well excited by a xenon arc lamp or dye-pumped lasers operating in the 720–750 nm range [6].

Alexa Fluor 680 (AF680) has a peak excitation at 679 nm and maximum emission at 702 nm. The dye is spectrally similar to the Cy5.5 dye. Fluorescence emission of the AF680 dye is also suitably different from that of other commonly used red fluorophores, such as the tetramethylrhodamine, Texas Red, R-phycoerythrin, Alexa Fluor 594, and Alexa Fluor 647 dyes [7].

Typically, the FRET fluorophore molecule pairs are characterized via the Förster radius (R_o), which is the distance at which the energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated). In this study, the well-characterized molecules, streptavidin and biotin, were labeled to the fluorophores in order to examine the percent energy transfer and Förster distance. The streptavidin biotin-binding protein binds four biotins per molecule with a high level of affinity and selectivity. Dissociation of biotin from streptavidin is reported to be fast, $2.4x10^{-6}$ s^{-1} [8].

Additionally, the potential as a NIR FRET glucose biosensor was examined using the established competitive binding technique of dextran and Con A. Dextran is an inhibitor to Con A. When glucose is added to the bound solution of dextran and Con A, glucose will displace the dextran and bind to the Con A. This displacement will take place due to the high affinity of glucose to Con A. As a result of the displacement, the change in FRET can be monitored.

The main objective of this investigation was to determine whether AF750 and AF680 is a viable FRET pair for implantable glucose biosensors. Three experiments were performed: 1) AF750 and AF680 were labeled to streptavidin and biotin and the energy transfer and Forster's distance was determined; 2) AF750 and AF680 were labeled to Con A and dextran (MW 3000 and MW 10,000) and glucose response was determined in a spectrofluorometer; and 3) AF750 and AF680 were labeled to Con A and dextran (MW 3000) and glucose response was determined through porcine skin. In previous experiments investigating dextran, researchers used large chains of dextran whose molecular weight ranged from 500,000 to 2,000,000 MW [4]. These experiments will help facilitate improvements in the development of an implantable biosensor used to monitor glucose.

2. Material and Methods

2.1. Material

Concanavalin A (5 mg/ml), sodium bicarbonate (84.01 MW, 0.1 M, pH 8.3), bovine serum albumin (2 mg/ml, pH 7.0), phosphate buffered saline (0.01M, pH 7.4), and beta-D(+) glucose (180.2 MW, 100 mg/ml) were purchased from Sigma (St. Louis, MO). Streptavidin-Alexa Fluor 680 (52,800 MW, 1 mg), Alexa Fluor 680-dextran (molecular weight 10,000 Da, 1 mol AF680/mol of dextran; molecular weight 3000 Da, 1 mol AF680/mol of dextran), Alexa Fluor 750 carboxylic acid (succinimidyl ester, 1 mg), and a FluoReporter Mini-biotin-XX protein labeling kit (5 labeling of 0.1-3 mg protein each) were purchased from Invitrogen (Carlsbad, CA). Dialysis tubing (6000-8000 MWCO) was purchased from Fisher Scientific (Hampton, NH).

2.2. Instrumentation

A UV-VIS absorbance spectrometer (Beckman DU 520) was used in order to collect absorbance spectra and perform catalytic activity tests. The slit size (4 nm) and scanning speed (595 nm/min) were held constant throughout all the experiments. A scanning fluorescence spectrometer (FluoroMax-3 Jobin Yvon, Hobira) was used to collect the fluorescence emission spectra by exciting samples at 680 nm. The slit size and integration time were 7 nm and 0.3 s, respectively.

2.3. Labeling Technique

BSA was diluted in 10 mM of a sodium phosphate buffer. Following the protocol outlined in the Invitrogen biotinylation kit, biotin was bound to BSA. Biotin was labeled with AF750 via the labeling kit protocol from Invitrogen.

A Bio-Rad experiment was conducted in order to determine the final protein concentration and the degree of labeling of the BSA-biotin. The final BSA-biotin concentration was 1.103 mg/ml and the degree of labeling (DOL) was determined through the use of UV-VIS spectrometry. The labeled BSA was scanned and the absorbance at 749 nm was determined. The DOL was calculated according to equation (1):

$$DOL = (A_{max} \times MW) / ([Protein] \times \epsilon_{dye}) , \quad (1)$$

where A_{max} is the absorbance value of AF750 at the absorption maximum wavelength. The DOL was determined to be 0.8; thus, for every mole of protein there was approximately a mole of dye.

The AF680-streptavidin DOL was 3.0.

Utilizing the protocol provided by Molecular Probes, Con A (10 mg) was labeled with AF750. Again, a Bio-Rad experiment was conducted to determine the final protein concentration and the degree of labeling. The final Con A concentration was 9.86 mg/ml and the degree of labeling was calculated according to equation (1). The DOL was 2.94 mole of dye/mole of Con A. The AF680-dextran DOL is 1 mole of AF680 for every mole of dextran.

2.3.1. FRET Experiments Using AF680 and AF750

The experiments were conducted in 1 ml cuvettes. The spectrofluorometer was adjusted to 1 nm increments with an integration time of 0.5 seconds. 1μg of BSA-AF 750 was scanned by setting the excitation at 750 nm and the slits at 5. FRET was determined by placing 1μg of AF680-streptavidin in a cuvette. PBS was added and a scan was conducted to acquire the sample background without AF750-BSA biotin. Next, 1μg AF750-BSA biotin was added to the cuvette and more spectra were obtained. Energy transfer from the AF680-streptavidin (donor) was determined. The final sample was 1 μg AF750-BSA biotin in 1 μg AF680-streptavidin in 1ml PBS. Dilution effects were taken into account during the experiments.

2.3.2. FRET Experiments via Dextran Size Testing for Glucose Response

AF680 and AF750 were used in the experiments for detecting glucose. As stated earlier, a competitive binding technique was utilized for the glucose sensor. Con A is considered to be a glucose binding protein. While dextran will bind to Con A, it has a lower affinity than glucose. By exploiting the lower binding affinity of dextran to Con A, the dextran will be replaced by the higher affinity glucose to Con A. This displacement will result in a change in FRET. In previous experiments investigating dextran, researchers used large chains of dextran whose molecular weight ranged from 500,000 to 2,000,000 MW [4]. In this research two smaller molecular weight dextrans will be used, dextran 10,000 MW and 3000 MW. Since the competitive binding technique depends on the binding of dextran to Con A, by choosing a smaller chain of labeled dextran, the distance between the labeled dextran site and labeled Con A site will be closer, and thus will have a greater effect on energy transfer.

In this experiment, we examined the feasibility of utilizing the AF680/AF750 NIR FRET pair as a glucose sensor via a competitive binding technique. The effect of dextran size on glucose detection was also determined.

The experiments were conducted using 4 ml cuvettes. The excitation was set at 680 nm, which is the

excitation wavelength for the AF680-dextran. The increment was set at 1 nm while the integration time was at 0.3 s. The slits were opened to 7. The test on the dextran (10,000MW) was conducted by placing 0.1 μg of AF680-dextran in a 4 ml cuvette. PBS was then added for a total volume of 3 ml. A scan of the sample was performed to acquire the background without the presence of AF750-Con A. Then 0.282 μg of AF750-Con A was added to the same cuvette. A scan of the sample was performed to determine the percent of energy transfer from the FRET pair. The mole ratio was maintained at 4 moles of dextran to 1 mole of Con A. The same test was conducted with the same mole ratio 4:1 by using the dextran (3000MW). 0.1 μg of AF680-dextran and 0.789 μg of AF750-Con A were placed in a 4ml cuvette. Similar scans were obtained.

After 2 hr incubation to allow binding of the dextran to Con A, each sample was tested for glucose response. The test consisted of four additions of glucose concentrations from 0.016 to 0.397 μM. Lower concentrations than clinically relevant levels of glucose were utilized in order to examine the limit of detection. Ten minutes was allowed for incubation with the glucose after each addition before scanning. These experiments were repeated four times in order to obtain statistical data.

2.3.3. Transmission Studies of FRET Glucose Sensors through Porcine Skin

The main objective of using NIR dye is to be able to detect signals from an implantable biosensor. Skin acts as a barrier between the sensor and the detector. By placing a porcine tissue in front of the cuvette holder, this mimicked the signal detection of a sensor through skin.

The biosensor probes from the previous experiments were prepared and then scanned. Various thicknesses of porcine skin between 1.3mm and 3.8mm were utilized by placing it in front of the detector. The relation between the tissue thickness and the fluorescence signal was determined.

3. Results and Discussion

3.1. Fluorescence Resonance Energy Transfer (FRET) Experiments

In the following graphs, the results of the FRET streptavidin/biotin experiments are shown. The scan of the maximum peak of 1ug SA-AF680 donor without the presence of the BSA-AF750 acceptor was conducted. It gave a maximum peak of 702 nm. This peak represents the maximum emission of the AF680 when the donor is not present in the sample.

Next, the sample was scanned with the presence of the acceptor. The excitation was set at 680 nm. Fig. 1 provided peak of the donor with the presence of the acceptor which was approximately 63624 cps at 702 nm.

The energy transfer was calculated using equation (2):

$$\% \text{ Energy transfer} = 1 - I_{DwA} / I_{/D} , \quad (2)$$

where I_D is the donor's intensity without the presence of the acceptor and I_{DWA} is the donor's intensity with the acceptor presence. The energy transfer was 95.76 %. The results provided conclusive observations of energy transfer in term of increase in the acceptor peak and decrease in the donor peak. Thus, AF680 and AF-750 were used in future experiments as an NIR FRET pair. The forster distance was 43 nm.

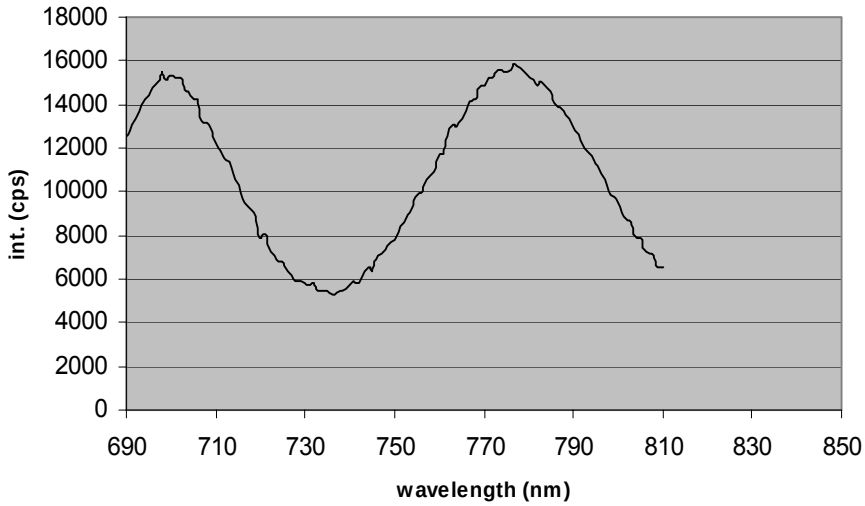


Fig. 1. Scan of SA-AF 680 with the presence of BSA Biotin-AF 750.

3.2. Dextran Size Effect on Glucose Response Experiments

Since the previous experiments determined that AF680 and AF750 fluorophores are viable FRET pairs, the next step was to utilize the fluorophores in a FRET-based glucose biosensor. Two different molecular weight dextrans were examined. The two different sizes, MW 10,000 and MW 3000, of the labeled dextran had similar concentrations and F/P ratios. In these experiments, four moles of dextran to one mole of Con A was maintained. This ratio increased the probability of dextran to bind to one or more of the four-sugar binding sites on Con A. Also a low glucose concentration was chosen to test the glucose response of the Con A with dextran 3000 MW and Con A with dextran 10,000 MW in order to determine the limit of detection.

Fig. 2 shows the average glucose response when the higher molecular weight dextran (10,000 MW) was utilized (n=4). A highly concentrated glucose solution was added to the curvettes to minimize any dilution effect. The data was plotted as Donor Acceptor ratio (D/A) vs. glucose concentration. For the first glucose addition of 0.016 μM, there was drop in the D/A peak ratio, but the response basically was a flat slope with additional glucose concentrations due to the low sensitivity.

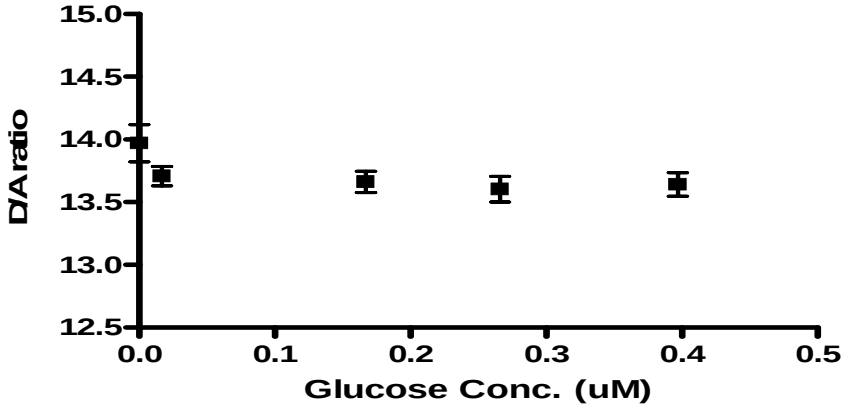


Fig. 2. Glucose Response of AF680-dextran (10,000MW) and AF750-Con A.

The average glucose response is shown for the 3000 MW dextran (n=4). Unlike the previous results using the higher molecular weight dextran (10000MW), there was an exponential decrease of the D/A

ratio as the glucose concentration was increased. The mathematical representation of the behavior of the response is shown as an exponential equation:

$$y = 14.076\, e^{-0.0069\, x}$$

The R^2 was 0.6831.

The smaller molecular weight dextran provided better sensitivity than the higher molecular weight dextran. It is possible that the poor glucose response when the larger dextran was utilized was due to its large sugar branches. Since there is no control over the labeled binding sites on the dextran molecules, the smaller dextran molecules may have a higher possibility of optimal labeling sites, which would place it in closer proximity to the labeled binding site on the Con A.

Additional experiments were performed with the lower molecular weight dextran. Fig. 3 shows the average results of the glucose probe, AF680-dextran (3000MW) and AF750-Con A, in a glucose concentration range between 3.33 mM and 33.65 mM (n=4). In these experiments, 0.2 µg of AF860-dextran with 2988 µl PBS was placed in a 4ml cuvette, and then 105.7 µg of Con A was added. These samples were incubated for 2 hours prior to glucose exposure.

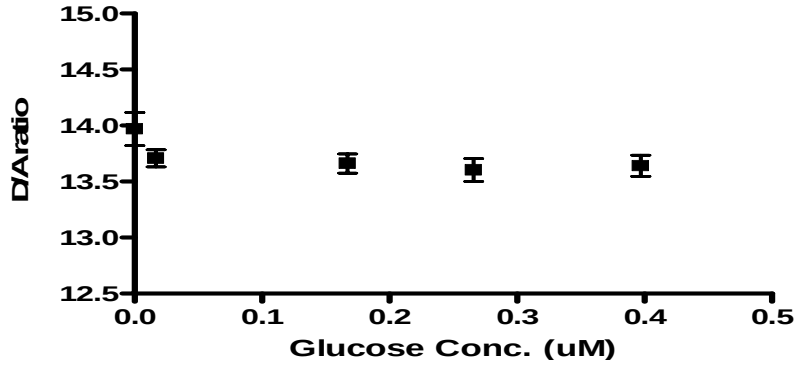


Fig. 3. Glucose Response of AF680-dextran (3000MW) and AF750-Con A.

Fig. 4 shows the exponential decrease to the peak of the D/A ratio as the glucose concentration increase.

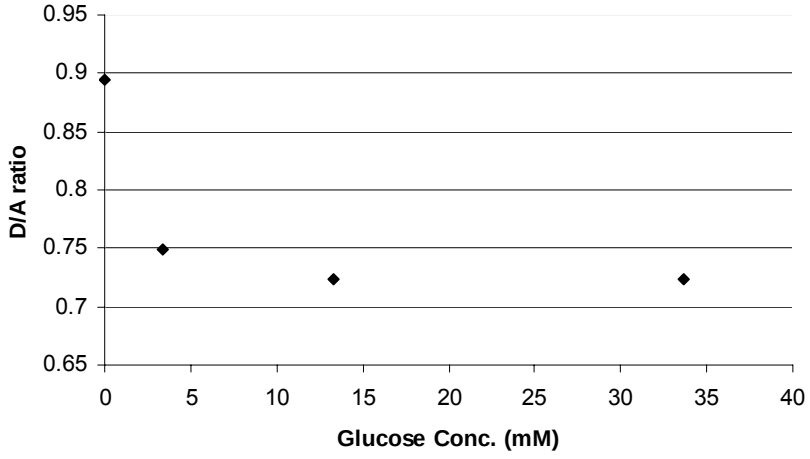


Fig. 4. Glucose Response of AF680-dextran (3000MW) and AF750-Con A with larger detection limits.

3.3. Results of the Transmission Studies of FRET Glucose Sensors through Porcine Skin

Implantable optical glucose sensors require the capture of transmitted fluorescence signals through skin. In this experiment various thicknesses of porcine skin was used to determine how much signal can be transmitted through it. The porcine skin was place between the cuvette holder and the detector. The results from testing different tissue thickness compared to the fluorescence captured showed a drop in the signal as the thickness of the tissue increases, as shown in Fig. 5.

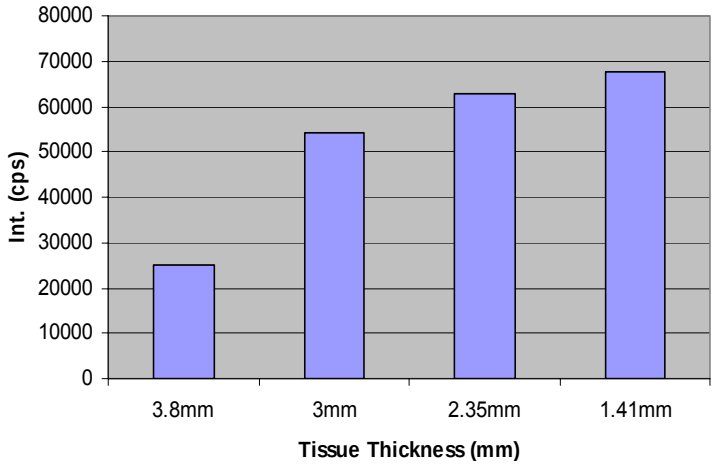


Fig. 5. The signal from different tissue thicknesses.

Fig. 6 represents the relationship between the transmitted signals vs. the tissue thicknesses. This relationship can be described mathematically by the following equation:

$$y = - 10045\ x^2 + 34922\ x + 37998$$

The R^2 value was 0.9926.

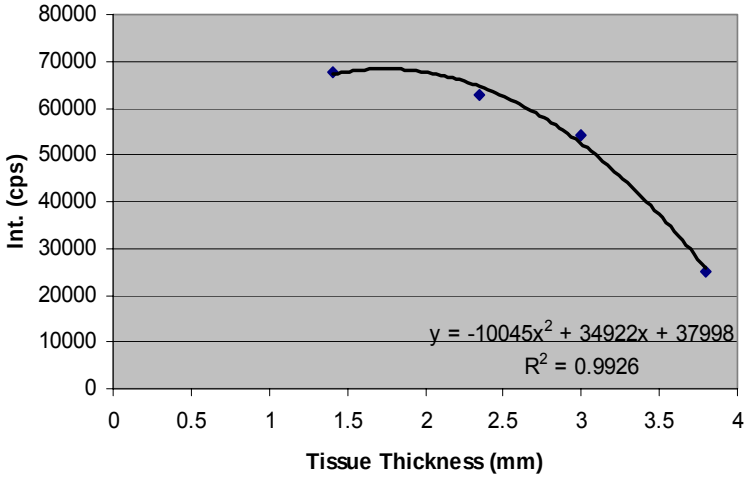


Fig. 6. Relation between tissue thickness and intensity.

These results imparted some important information and will be useful in the final *in vivo* sensor probe design. The placement of the light source and detector will be extremely important. These components

need to be placed in a location where the skin thickness is minimal. The light source should be powerful enough to penetrate the skin and elicit a strong fluorescence response that can be captured by the detector.

4. Conclusions

FRET is an optical ratiometric technique. It was used successfully in developing a transduction system to detect glucose *in vivo*. *In vivo* optical signal detection requires NIR dye. AF680 and AF750 were selected as NIR dyes that could operate with in the biological window. They have been tested to ensure their functionality as a FRET pair. The results confirmed that AF680 and AF750 is a FRET pair. The energy transfer was 95.76 %. Competitive binding technique between protein Con A and the two substrates, dextran and glucose was used. After forming the complex of labeled Con A and labeled dextran binding between dextran and Con A has taken place. When glucose was added with different concentration, a change to the background signal happened. This change in signal was due to glucose higher affinity to Con A than dextran. Further testing on two different molecular size of dextran, 10000 MW and 3000 MW, was conducted. The results of dextran with 3000 MW provided better FRET signal than dextran 10000 MW.

In the testing process, the chosen detection range of glucose concentration was adjusted to cover the upper limit of the normal blood glucose concentration in humans.

Finally, detecting of peak signal through tissue was possible but only 1.92% of that intensity was captured at the 3.8 mm skin thickness. When the tissue thickness was reduced then the captured signal increased. All the above results can be utilized in designing more sensitive *in vivo* optical glucose biosensor with superior sensitivity.

Acknowledgments

The authors gratefully acknowledge funding from the Missouri F21C and the Bioprocessing and Biosensor Center.

References

[1]. http://www.diabetes.org/home.jsp (4-13-2008).
[2]. J. Wang, Bismuth-coated screen-printed electrodes for stripping voltammetric measurements of trace lead, *Ekectroanalysis*, 13, 2001, pp. 13-16.
[3]. N. Wisniewski, F. Moussy, and W. M. Reichert, Characterization of implantable biosensor membrane biofouling, *Fresenius Journal of Analytical Chemistry*, 366, 2000, pp. 611-621.
[4]. P. S. Grant, McShane, M. J., Development of Multilayer Fluorescent Thin Film Chemical Sensors Using Electrostatic Self Assembly, *IEEE Sensors Journal*, 3, 2003, pp. 139-146.
[5]. D. J. S. Birch and O. Res. Rolinski, *Chem. Intermed.*, 27, 2001, p. 425.
[6]. Alexa Fluor 750 http://probes.invitrogen.com/servlets/spectra?fileid=21037p72 (4-13-2008).
[7]. Alexa Fluor 680 http://probes.invitrogen.com/servlets/spectra?fileid=21057p72 (4-13-2008).
[8]. U. Piran and WJ. Riordan, Dissociation rate constant of the biotin-streptavidin complex, *J Immunol Methods*, 133, 1990, p. 141.

Guide for Contributors

Aims and Scope

Sensors & Transducers Journal (ISSN 1726-5479) provides an advanced forum for the science and technology of physical, chemical sensors and biosensors. It publishes state-of-the-art reviews, regular research and application specific papers, short notes, letters to Editor and sensors related books reviews as well as academic, practical and commercial information of interest to its readership. Because it is an open access, peer review international journal, papers rapidly published in *Sensors & Transducers Journal* will receive a very high publicity. The journal is published monthly as twelve issues per annual by International Frequency Association (IFSA). In additional, some special sponsored and conference issues published annually. *Sensors & Transducers Journal* is indexed and abstracted very quickly by Chemical Abstracts, IndexCopernicus Journals Master List, Open J-Gate, Google Scholar, etc.

Topics Covered

Contributions are invited on all aspects of research, development and application of the science and technology of sensors, transducers and sensor instrumentations. Topics include, but are not restricted to:

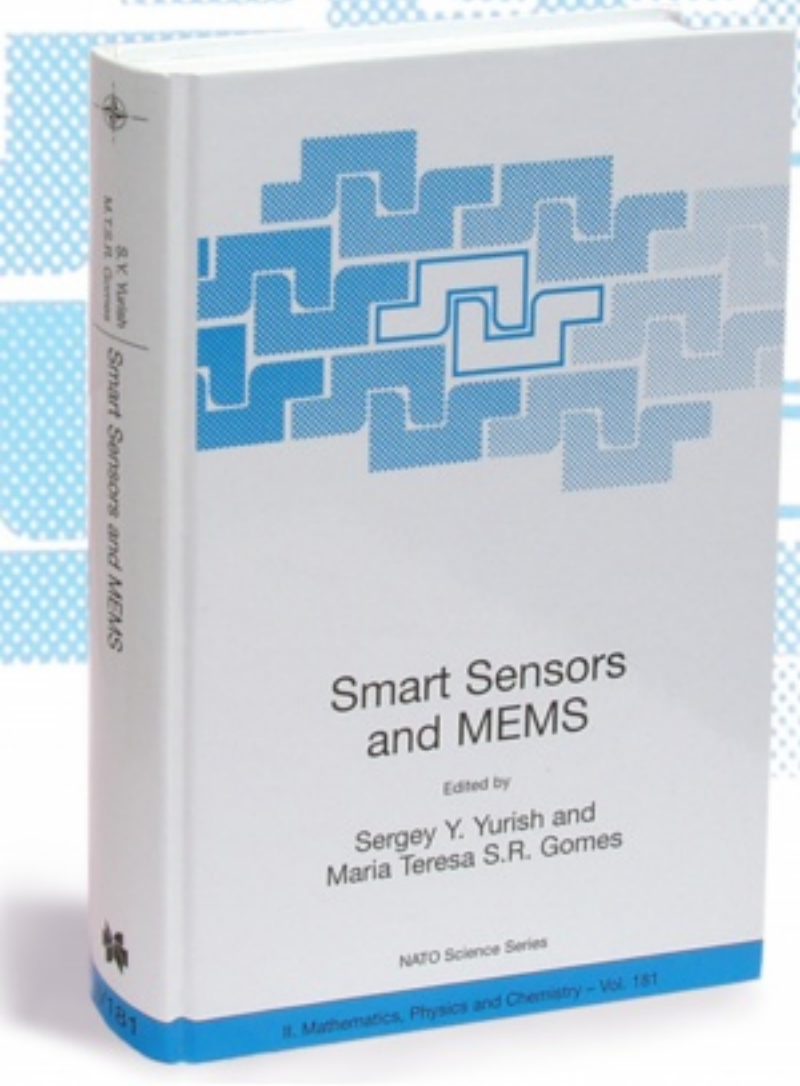
- Physical, chemical and biosensors;
- Digital, frequency, period, duty-cycle, time interval, PWM, pulse number output sensors and transducers;
- Theory, principles, effects, design, standardization and modeling;
- Smart sensors and systems;
- Sensor instrumentation;
- Virtual instruments;
- Sensors interfaces, buses and networks;
- Signal processing;
- Frequency (period, duty-cycle)-to-digital converters, ADC;
- Technologies and materials;
- Nanosensors;
- Microsystems;
- Applications.

Submission of papers

Articles should be written in English. Authors are invited to submit by e-mail editor@sensorsportal.com 8-14 pages article (including abstract, illustrations (color or grayscale), photos and references) in both: MS Word (doc) and Acrobat (pdf) formats. Detailed preparation instructions, paper example and template of manuscript are available from the journal's webpage: http://www.sensorsportal.com/HTML/DIGEST/Submition.htm Authors must follow the instructions strictly when submitting their manuscripts.

Advertising Information

Advertising orders and enquires may be sent to sales@sensorsportal.com Please download also our media kit: http://www.sensorsportal.com/DOWNLOADS/Media_Kit_2009.pdf



Smart Sensors and MEMS

Edited by

Sergey Y. Yurish and
Maria Teresa S.R. Gomes

The book provides an unique collection of contributions on latest achievements in sensors area and technologies that have made by eleven internationally recognized leading experts ...and gives an excellent opportunity to provide a systematic, in-depth treatment of the new and rapidly developing field of smart sensors and MEMS.

The volume is an excellent guide for practicing engineers, researchers and students interested in this crucial aspect of actual smart sensor design.

Kluwer Academic Publishers