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Microporated PEG Spheres for Fluorescent Analyte Detection

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Abstract Poly(ethylene glycol) (PEG) hydrogels have been used to encapsulate fluorescently labeled molecules in order to detect a variety of analytes. The hydrogels are designed with a mesh size that will retain the sensing elements while allowing for efficient diffusion of small analytes. Some sensing assays, however, require a conformational change or binding of large macromolecules, which may be sterically prohibited in a dense polymer matrix. A process of hydrogel microporation has been developed to create cavities within PEG microspheres to contain the assay components in solution. This arrangement provides improved motility for large sensing elements, while limiting leaching and increasing sensor lifetime. Three hydrogel compositions, 100% PEG, 50% PEG, and microporated 100% PEG, were used to create pH-sensitive microspheres that were tested for response time and stability. In order to assess motility, a second, more complex sensor, namely a FITC-dextran/TRITC-Con A glucose-specific assay was encapsulated within the microspheres.

Keywords Poly(ethylene glycol) · PEG · Microporation · Fluorescence · Mobility · Porosity

Introduction

Polymer hydrogels have been used to encapsulate fluorescently labeled molecules to sense a variety of analytes [1–7]. Poly(ethylene glycol) (PEG) is one such polymer that is favored for biosensing because of its biocompatibility [8–11]. PEG hydrogels are created via crosslinking of the polymer chains, creating a mesh that allows for efficient diffusion of small analytes while retaining larger molecules. The mesh size of a gel synthesized from pure poly(ethylene glycol) diacrylate (PEG-DA) has been reported between 8.6 and 10.8 Å [12–14]. Mesh size of the hydrogel can be increased through introduction of aqueous solution to the precursor solution, reducing the potential for crosslinking. The ratio of aqueous solution to PEG-DA has been shown to alter the mesh size considerably [13]. The addition of aqueous solution in turn alters the mechanical properties of the polymer and also requires continuous hydration after formation of the hydrogel to prevent drying. This increase in mesh size does allow for increased diffusion of small analytes through the hydrogel [12]. However, in applications focusing on entrapping larger molecules within the hydrogel matrix, increased leeching typically results, thus limiting the life span of the sensor.

For assays that detect a spatial displacement of molecules that bind to the sensing element inducing a change in fluorescence through a fluorescence resonance energy transfer (FRET) reaction or steric quenching, the current hydrogels do not allow for sufficient mobility of the components without increased leaching. For example, several approaches for fluorescent glucose sensing have been successful in solution, but lose activity upon hydrogel encapsulation because of the physical inability of the assay chemistry to completely dissociate and reassociate [3, 15]. One such sensor is based on a FRET reaction that occurs between fluorescein isothiocyanate (FITC)-labeled dextran and tetramethylrhodamine isothiocyanate (TRITC)-labeled concanavalin A (Con A) that are within close proximity, as the absorption spectra of TRITC heavily overlaps the emission spectrum of FITC

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[16–18]. Con A, a lectin derived from the Jack bean plant, has an inherent affinity for saccharides such as dextran and glucose [19, 20]. The sensor is created by allowing labeled dextran to bind with the labeled Con A, bringing the independent fluorophores within 50 Å, close enough to cause significant quenching of the FITC signal [18]. As glucose is added, dextran is displaced, increasing the distance between the fluorophores, thereby lowering the quenching and increasing the signal intensity. This process has been shown to be reversible, meaning with loss of glucose in the system, the dextran molecule will resume conjugation with the Con A protein and the FITC fluorescence will again be quenched. In attempt to move this chemical assay into a protected environment where it could eventually be implanted into the human body, PEG encapsulation was investigated [3, 21]. Pure 100% PEG was disregarded because of the limited motility it offered to the entrapped molecules. Higher hydration ratios have been investigated, but they resulted in leeching of the sensor chemistry. Using a heterofunctional derivative of poly (ethylene glycol), the sensing elements were cross-linked to the backbone of the polymer matrix with the freedom to bind and unbind. This procedure showed promise, but resulted in a large reduction in the response of the sensor due to the decreased motility offered by the tethering ester complex [3].

Several attempts to improve sensor response have been made using various hollow embodiments. Ballerstadt and Schultz created a fluorescence affinity hollow fiber sensor based on the competitive binding of fluorophore-labeled Con A to glucose residues inside Sephadex beads [22, 23]. As glucose diffuses into the sensor, the Con A molecules are displaced from the beads, and being no longer shielded from the excitation light, display an increase in fluorescence intensity. The main problem with this sensor was leakage of the Con A through the sealant/membrane interface, reducing sensor lifetime and exposing the surrounding tissues to potentially toxic Con A. Barone, Parker, and Strano proposed a single-walled carbon nanotube-based (SWNT) optical sensor that is coated in a hydrogel matrix to improve biocompatibility and prevent leakage [24]. SWNTs fluoresce from 900 to 1600 nm and are coated with a glucose analogue like dextran. A glucose-specific protein such as Con A binds to the dextran coated walls, attenuating the fluorescence signal, until glucose is introduced, competitively binding and freeing the Con A. This is potentially advantageous because SWNTs are photobleaching-resistant, but results so far have been limited to theoretical modeling. Chinnayelka and McShane have created microcapsules containing a competitive binding assay by using a layer-by-layer fabrication technique [25, 26]. Initially, dissolvable resin microparticles were coated with FITC-dextran and TRITC-Con A layers and an outer polymer layer [25]. Then the core was dissolved to produce a hollow microcapsule lined with immobilized Con A and dextran. This approach had a limited response due to the low assay concentration within the walls and a significant drop in pH was used to dissolve the inner core that could be harmful to many sensing assays. This system was improved upon by first creating hollow spheres and then filling them with a competitive binding assay that was free to move within the microcapsules [26]. This method is promising, but the loading of the microcapsules is diffusion-limited and requires excessive amounts of expensive assay components to drive the particles into the capsules.

Our group has devised a simple approach of hydrogel microporation of PEG microspheres in order to increase assay motility while preventing assay leaching. A variety of methods exist for creating porous hydrogels, including the porosigen technique, phase separation technique, foaming technique, and crosslinking of individual hydrogel particles [27]. The porosigen or porogen technique is particularly useful for our applications as it can be used in many different polymers, is capable of creating a variety of pore sizes, and can create individual cavities as opposed to channels that open to the surface. A water-soluble porogen material, such as sucrose, is dispersed throughout the hydrogel. After polymerization, the porogen is dissolved and dispersed, leaving cavities that are dependent on the size of the initial sucrose crystals [28]. Similar processes have been used previously for drug delivery and tissue engineering, but have been focused primarily on transporting rather than retaining molecules [29, 30]. Applied to sensing, this process creates small cavities within the hydrogel that contain the assay component in aqueous solution. The cavities allow for a response similar to that in pure aqueous solution by providing more room for increased assay component motility, while simultaneously reducing leaching through the fine mesh of the 100% PEG hydrogel.

Materials and methods

Materials

Liquid poly(ethylene glycol) diacrylate (PEG-DA) with an average molecular weight of 575, dextran-bound fluorescein isothiocyanate (FITC-dextran), D-glucose, D-mannitol, light mineral oil, 0.1 M phosphate buffered saline (PBS), and 1 M hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO). Tetramethylrhodamine isothiocyanate concanavalin A (TRITC-Con A) was purchased from Molecular Probes (Eugene, OR). 2-Hydroxy-2-methyl-1-phenyl-1-propanone (Darocur® 1173) was obtained from Ciba Specialty Chemicals (Tarrytown, NY). The α-acryloyl, ω-*N*-hydroxysuccinimidyl ester of poly(ethylene glycol)-propionic acid (PEG NHS, MW 3400) was purchased from Nektar (Huntsville, AL). All aqueous experiments were performed with deionized water with a resistance of 18 M$\Omega \cdot$ cm (Millipore, Billerica, MA).

Sphere fabrication

Three hydrogel compositions were created for pH sensing: 100% PEG, 50% aqueous PEG, and microporated 100% PEG. The pure PEG hydrogel solutions were composed of liquid PEG-DA while the aqueous hydrogels were 50% PEGDA and 50% water. A pH-sensitive solution of 10 mg/mL FITC-dextran (MW 10,000, 1% v/v) was added. Darocur, a photoinitiator, was added to promote cross-linking during polymerization (1% v/v). Spherical particles were created using an emulsion technique and cured using UV exposure for 2.0 s (300 mW/cm^2, $\lambda_{peak} = 365$ nm, EFOS Ultracure 100SS Plus).

For glucose sensing, the same hydrogel compositions were used with a previously devised FITC-dextran/TRITC-Con A assay [17, 18]. An additional hydrogel composition was also created with the TRITC-Con A chemically immobilized through linkage to PEG-NHS, as done in previous work [3].

In order to induce microporation, a powdered combination of mannitol and fluorophore was added to the pure PEG solution. The powders were formed through lyophilization of 100 mg of mannitol, 100 μL of fluorophore assay solution, and 900 μL of 0.1 M PBS. Large aggregations of the powder were separated by sonicating the PEG solution for 15 min. After UV polymerization, the mannitol porogen was dissolved and washed out of the PEG hydrogel with PBS, leaving pockets containing the sensing elements in aqueous solution.

Detection and imaging

A fluorescence detection system from Photon Technologies Inc. (PTI, Lawrenceville, NJ) was used to collect fluorescence at 90°. The system consisted of a mercury arc lamp, an excitation monochromater, a sample chamber, an emission monochromater, and a PMT. The spheres were placed in a microcuvette (Ocean Optics, Dunedin, FL) with 0.1 M PBS and excited with 480 nm light. Spectra were acquired from 500 to 550 nm for pH sensing and from 500 to 600 nm for glucose sensing to include the FITC peak around 520 nm and the TRITC peak at approximately 580 nm. Changes were induced in pH using 1 M HCl and verified using a pH meter (420 A+, Orion; electrode 5990-30, Cole-Parmer, Vernon Hills, IL).

Fluorescent images were acquired using an inverted microscope (Nikon CFI60, East Rutherford, NJ) containing two emission filters and differential interface contrast (DIC) optics (Chroma DIC, FITC, Filter Sets, Rockingham, VT). Fluorescent and DIC images were acquired using standard light microscopy (MetaVue Software, Sunnyvale, CA) and a CCD camera (Roper Scientific Photometrics Coolsnap HQ, Duluth, GA) at 100 ms integration. Confocal microscopy was implemented to determine the degree of poration using 2D fluorescent images at the point of maximum diameter of the sphere. Using the image processing toolbox with Matlab, a program was generated which calculated the fraction of fluorescent pixels to background pixels arriving at an estimation of percent poration.

Results

Sphere characterization

FITC fluorophores were encapsulated in a PEG-DA hydrogel to determine the feasibility of a new microporated sphere moiety. FITC undergoes a change in fluorescence intensity in the presence of H^+ ions and was therefore used to sense pH. The average sphere diameter was found to be about 370 μm for a set of 60 spheres made in 3 batches. The optimal sphere size has been previously determined to be 100 μm based on photon flux and fluorescence intensity, but may be larger due to the additional light scattering from the cavities [31].

Fluorescent images, generated by confocal microscopy, of the microspheres were taken on 52 individual spheres. The number of fluorescent pixels (given a threshold) was counted and divided by the total sphere pixel area to yield a percent poration for each sphere (Fig. 1). The percent pore volume was estimated using image analysis and found to be around 15.12% with a standard deviation of 2.24%. Figure 2 shows a sphere with micropores (a) and the location of the fluorophore within the PEG (b). The fluorophore is mainly contained within the cavities of the microporated spheres, easily detectible, but less prone to surface leaching.

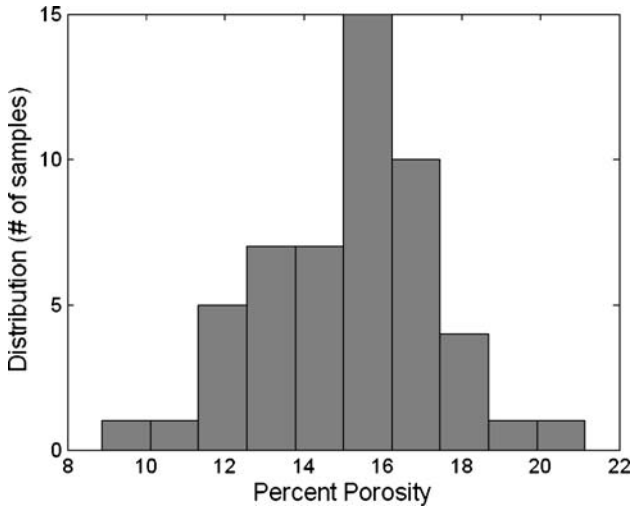

Fig. 1 The distribution of sphere poration. The poration is dependent on the amount of porogen included in the hydrogel precursor solution. The percent pore volume was estimated using image analysis and was found to be approximately 15.12% with a standard deviation of 2.24%

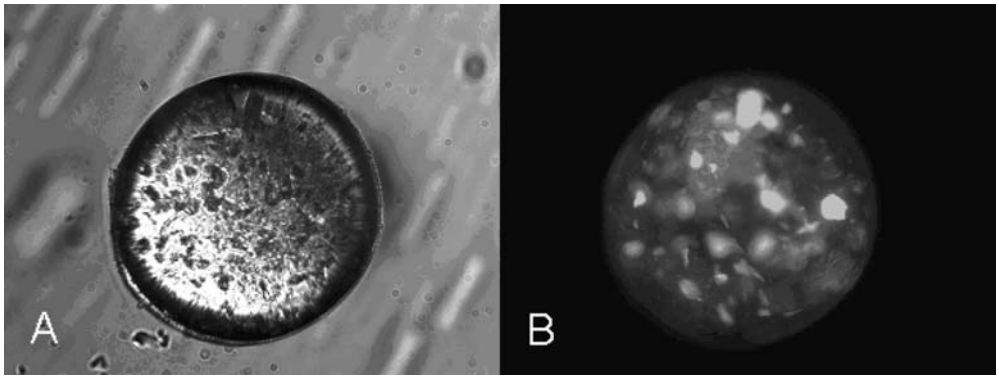

Fig. 2 A sphere of approximately 400 μm diameter with micropores (a) and fluorophore contained within the cavities distributed throughout the sphere (b). These images show that the spheres are spherical in nature and that the fluorescent pores are well distributed throughout the sphere. The lack of definition of the fluorescent pores is due to out of plane fluorescence adding to the image

Time response

The diffusion coefficient through a hydrogel decreases as the cross-linking density increases [32]. Analytes should therefore diffuse more quickly through the 50% PEG than through the 100% PEG, and much faster in free solution where there are few barriers. The microporated spheres were designed to decrease the time response by alternating areas of dense polymer and solution. Combining the two environments to create a pure PEG sphere with 15% poration, should result in a noticeable decrease in small particle travel time.

The time response of the sphere sensors was measured by inducing a change in pH. A steady baseline intensity was established and then 5 μL of HCl was added. Figure 3 shows the nearly immediate response in solution that is fast enough to capture the initial buffer activity. The time response of pure PEG spheres is much slower, but microporation improves the reaction time, nearly matching that of the 50% PEG hydrogel. This could potentially be further improved by reducing the sphere size and increasing the percentage of pore volume within the microporated spheres.

Sensor stability

Sensor stability was determined using spectra taken daily for one month. Prior to measurement, the buffer solution surrounding the spheres was removed and fresh PBS was added so that fluorophore that was initially on the sphere surface and hence fell into solution would be excluded from the intensity readings. Intensities for each sphere composition were normalized for comparison and Shewhart control charts were created using JMP, a statistical software package, as shown

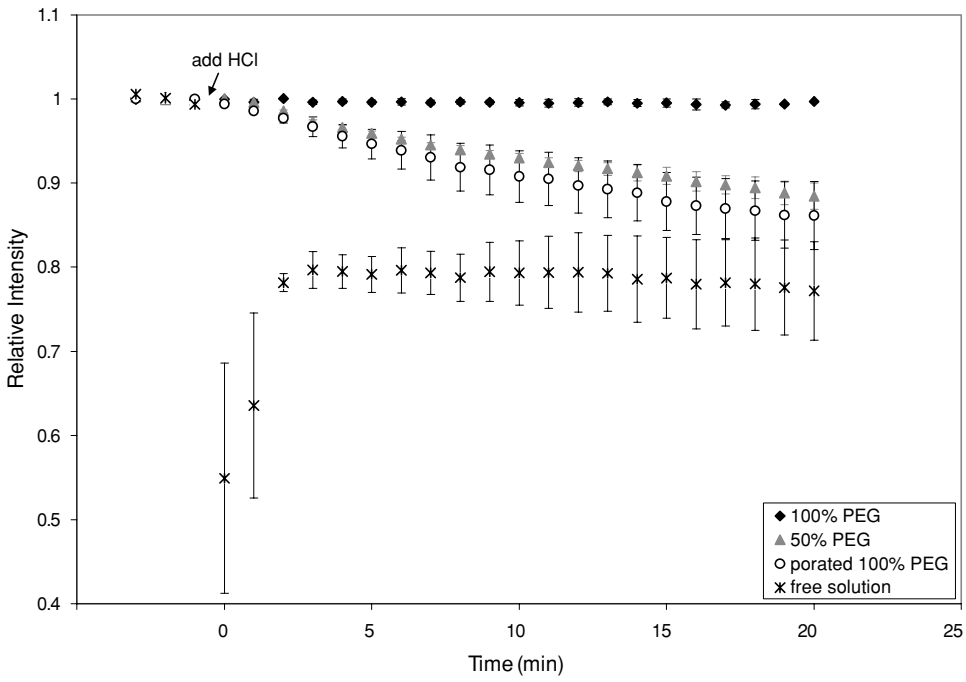

Fig. 3 Sensor time response to a pH change induced by 5 μL of HCl in the three sphere sets versus free FITC-dextran in solution. It can be seen that the 100% PEG spheres do not elicit a large change in signal intensity while the free solution responds so quickly that the buffer response is observed. From this data, it appears that the porated microspheres respond similarly to the 50% spheres, both of which are about half as responsive as FITC in solution

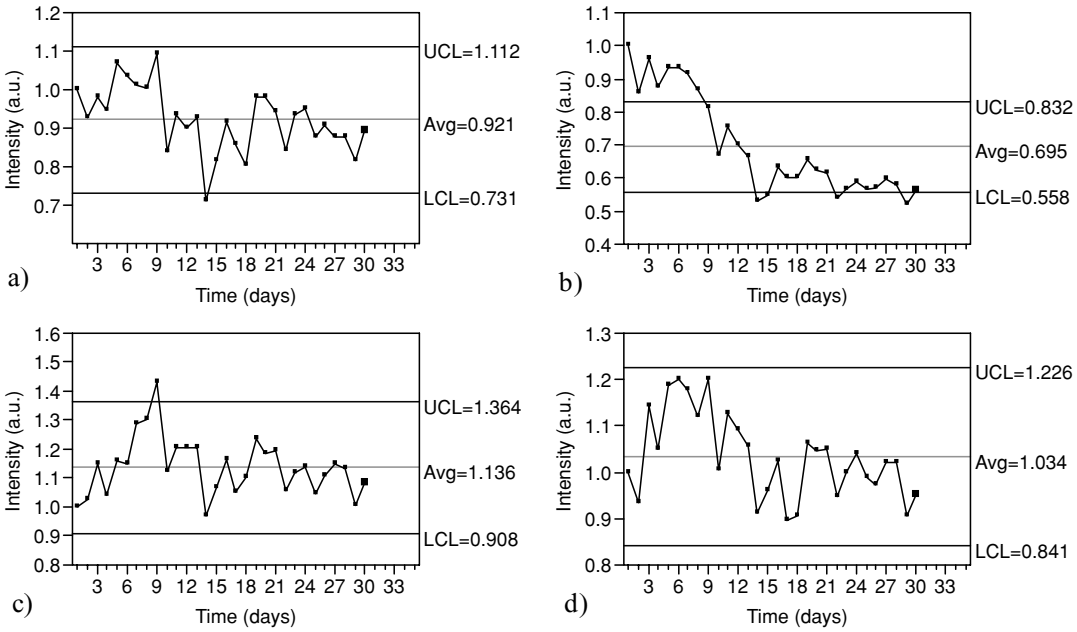

Fig. 4 Control charts for sensor stability over one month showing the upper (UCL) and lower control limits (LCL). (a) 100% PEG (b) 50% PEG (c) porated 100% PEG (d) free solution. Only the 50% PEG experiences a statistically significant decrease in intensity due to fluorophore leaching

in Fig. 4 (Individual Measurement Chart, JMP 6, SAS Institute, Cary, NC) [33, 34]. The upper (UCL) and lower control limits (LCL) are defined as:

$$UCL = \bar{X} + k\sigma$$
$$LCL = \bar{X} - k\sigma \quad (1)$$

where $\bar{X}$ is the mean of the individual measurements, k is a multiple of the standard error set at 3, and σ is the standard deviation [33]. The 100% PEG spheres remained relatively stable as the fine mesh size limits fluorophore leaching and promotes sensor stability over time. The 50% PEG spheres, on the other hand, showed a large decrease in intensity attributed to significant fluorophore leaching. The porated 100% PEG spheres performed well, remaining relatively stable throughout the month as the dense polymer matrix retained the majority of the sensing molecules. The free FITC in solution shows slight day to day variability potentially due to a combination of source variability, temperature fluctuation, photobleaching, and changes in sphere packing. System noise is similarly evident in the three sphere sets, but the overall statistical trends are notable.

Sensor motility

Sensor motility was tested using a glucose sensitive assay that depends on a binding reaction with FITC-dextran and TRITC-Con A. A 1000 mg/dL glucose change was induced and the resulting change in fluorescence intensity was measured (Fig. 5). The response was minimal in the 100% PEG spheres and the 50% PEG spheres, most likely because of the immobilization of the assay components within the PEG mesh. The PEG-NHS spheres used in previous research have a portion of the sensor chemistry bound directly to the polymer to minimize leaching, which also appeared to reduce the sensor activity in combination with the limiting mesh size of the hydrogel backbone [3]. The response in the microporated spheres was, however, much faster than that of the other sphere sets. It was also only slightly slower than the response in solution and comparable in relative intensity. This undiminished response is because the competitive binding event is essentially occurring within an aqueous microenvironment unhindered by the hydrogel mesh, though the hydrogel mesh does surround the microenvironment and prevent leaching.

Conclusion

Microporation of poly(ethylene glycol) spheres creates cavities where analytes may quickly diffuse and interact with the sensing elements. Pure 100% PEG has a very slow response time, which is significantly improved by microporation, nearly matching the response of 50% PEG hydrogels. Loose mesh sizes promote fast analyte diffusion, but also tend to allow the assay chemistry to leach out into solution. Microporation allows for the use of a fine mesh size to minimize leaching without limiting molecule mobility. Over a one month period, the microporated spheres maintained reasonably consistent intensity levels, while the 50% PEG

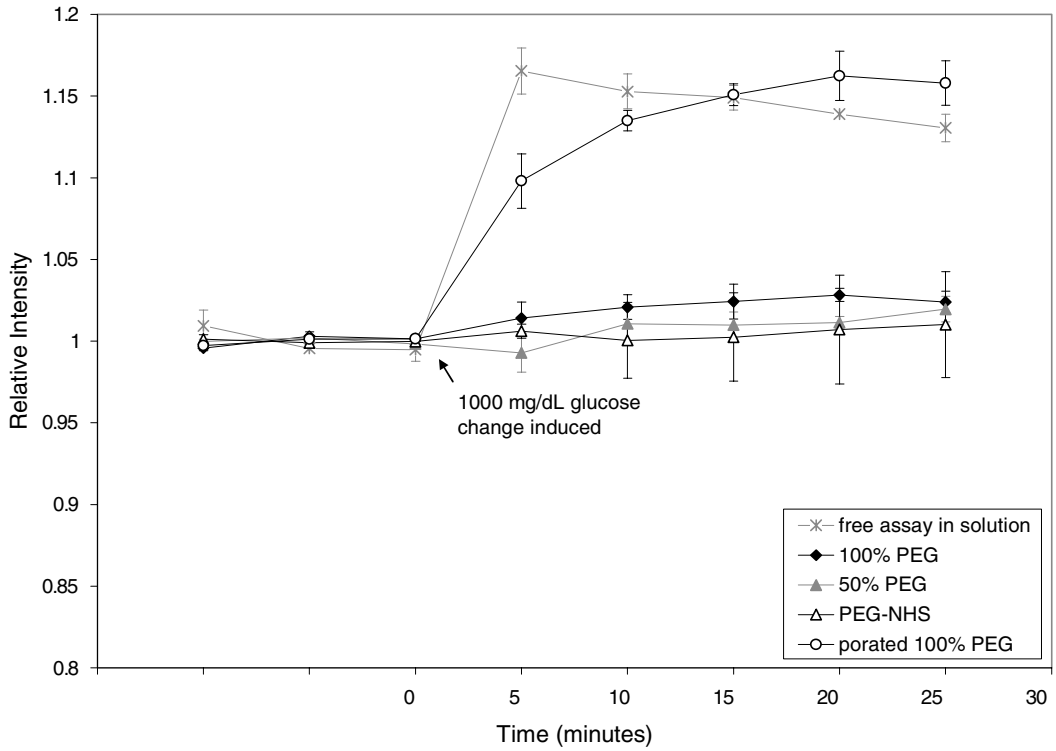

Fig. 5 Sensor response to 1000 mg/dL glucose change. It can be seen that the glucose response is minimal in the 100% PEG and 50% PEG compositions, presumably due to significant immobilization of the sensor components. The PEG-NHS configuration reported in previous publications also appeared to diminish the functionality of the glucose assay. Of most interest is that the response is maintained in the porated spheres as theorized

spheres experienced a significant decrease in fluorescence over time. Additionally, the microporated PEG provided adequate space for increased assay motility, allowing a binding reaction with FITC-dextran and TRITC-Con A to occur nearly unhindered while the other sphere compositions displayed minimal responses. Microporation of PEG creates cavities of aqueous solution in which the assay chemistry is free to react with small analytes, while limiting leaching, promoting both functionality and stability of the encapsulated sensors.

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Refrences

1. Yadavalli VK, Koh WG, Lazur GJ, Pishko MV (2004) Microfabricated protein-containing poly(ethylene glycol) hydrogel arrays for biosensing. Sensors Actuators B Chem 97(2–3):290–297
2. O'Neal DP, Meledeo MA, Davis JR, Ibey BL, Gant VA, Pishko MV, Coté GL (2004) Oxygen sensor based on the fluorescence quenching of a ruthenium complex immobilized in a biocompatible poly(ethylene glycol) hydrogel. IEEE Sensors J 4(6):728–734
3. Russell RJ, Pishko MV, Gefrides CC, McShane MJ, Coté GL (1999) A fluorescence-based glucose biosensor using concanavalin A and dextran encapsulated in a poly(ethylene glycol) hydrogel. Anal Chem 71(15):3126–3132
4. Kim SH, Kim B, Yadavalli VK, Pishko MV (2005) Encapsulation of enzymes within polymer spheres to create optical nanosensors for oxidative stress. Anal Chem 77(21):6828–6833
5. Russell RJ, Pishko MV, Simonian AL, Wild JR (2000) Poly(ethylene glycol) hydrogel-encapsulated fluorophore-enzyme conjugates for direct detection of organophosphorus neurotoxins. Abstracts of Papers of the American Chemical Society 219:U107–U107
6. Zguris J, Pishko MV (2005) pH sensitive fluorescent poly(ethylene) glycol hydrogel microstructures for monitoring in cell culture systems. Sens Lett 3(3):206–210
7. Heo J, Crooks RM (2005) Microfluidic biosensor based on an array of hydrogel-entrapped enzymes. Anal Chem 77(21):6843–6851
8. Sharma S, Johnson RW, Desai TA (2003) Ultrathin poly(ethylene glycol) films for silicon-based microdevices. Appl Surf Sci 206(1–4):218–229
9. Harris JM, Zalipsky S (1997) Poly(ethylene glycol): Chemistry and Biological Applications, in ACS Symposium Series, vol 680. American Chemical Society, Washington, DC, p 489
10. Rihova B (1996) Biocompatibility of biomaterials: Hemocompatibility, immunocompatibility and biocompatibility of solid polymeric materials and soluble targetable polymeric carriers. Adv Drug Deliv Rev 21(2):157–176

11. Quinn CAP, Connor RE, Heller A (1997) Biocompatible, glucose-permeable hydrogel for in situ coating of implantable biosensors. Biomaterials 18(24):1665–1670
12. Russell RJ, Axel AC, Shields KL, Pishko MV (2001) Mass transfer in rapidly photopolymerized poly(ethylene glycol) hydrogels used for chemical sensing. Polymer 42(11):4893–4901
13. Mellott MB, Searcy K, Pishko MV (2001). Release of protein from highly cross-linked hydrogels of poly(ethylene glycol) diacrylate fabricated by UV polymerization. Biomaterials 22(9):929–941
14. Kurdikar DL, Peppas NA (1995) The volume shrinkage, thermal and sorption behavior of polydiacrylates. Polymer 36(11):2249–2255
15. Ibey BL, Beier HT, Yadavalli VK, Rounds RM, Coté GL, Pishko MV (2005) Competitive binding assay for glucose based on glycodendrimer-fluorophore conjugates. Anal Chem 77(21):7039–7046
16. Schultz JS, Mansouri S, Goldstein IJ (1982) Affinity sensor – a new technique for developing implantable sensors for glucose and other metabolites. Diabetes Care 5(3):245–253
17. Meadows D, Schultz JS (1988) Fiber-optic biosensors based on fluorescence energy-transfer. Talanta 35(2):145–150
18. Meadows DL, Schultz JS (1993) Design, manufacture and characterization of an optical-fiber glucose affinity sensor-based on an homogeneous fluorescence energy-transfer assay system. Anal Chim Acta 280(1):21–30
19. Bittiger H, Schnebli HP (1976) Concanavalin a as a tool. John Wiley & Sons, London
20. Chowdhury TK, Weiss AK (1975) Concanavalin A, in Advances in experimental medicine and biology, vol 55, 2nd edn. Plenum Press, New York
21. Ibey BL, Meledeo MA, Gant VA, Yadavalli V, Pishko MV, Cote GL (2003) In vivo monitoring of blood glucose using poly(ethylene glycol) microspheres. Proc SPIE 49651-6
22. Ballerstadt R, Schultz JS (2000) A fluorescence affinity hollow fiber sensor for continuous transdermal glucose monitoring. Anal Chem 72(17):4185–4192
23. Ballerstadt R, Polak A, Beuhler A, Frye J (2004) In vitro long-term performance study of a near-infrared fluorescence affinity sensor for glucose monitoring. Biosens Bioelectron 19(8):905–914
24. Barone PW, Parker RS, Strano MS (2005) In vivo fluorescence detection of glucose using a single-walled carbon nanotube optical sensor: Design, fluorophore properties, advantages, and disadvantages. Anal Chem 77(23):7556–7562
25. Chinnayelka S, McShane MJ (2004) Glucose-sensitive nanoassemblies comprising affinity-binding complexes trapped in fuzzy microshells. J Fluorescence 14(5):585–595
26. Chinnayelka S, McShane MJ (2005) Microcapsule biosensors using competitive binding resonance energy transfer assays based on apoenzymes. Anal Chem 77(17):5501–5511
27. Chen J, Park H, Park K (1999) Synthesis of superporous hydrogels: Hydrogels with fast swelling and superabsorbent properties. J Biomed Mater Res 44(1):53–62
28. Oxley HR, Corkhill PH, Fitton JH, Tighe BJ (1993) Macroporous hydrogels for biomedical applications: methodology and morphology. Biomaterials 14(14):1064–1072
29. Badiger MV, McNeill ME, Graham NB (1993) Porogens in the preparation of microporous hydrogels based on poly(ethylene oxides). Biomaterials 14(14):1059–1063
30. Filmon R, Retailleau-Gaborit N, Grizon F, Galloyer M, Cincu C, Basle MF, Chappard D (2002) Non-connected versus interconnected macroporosity in poly(2-hydroxyethyl methacrylate) polymers. An X-ray microtomographic and histomorphometric study. J Biomater Sci Polym Ed 13(10):1105–1117
31. Yadavalli VK, Russell RJ, Pishko MV, McShane MJ, Coté GL (2005) A Monte Carlo simulation of photon propagation in fluorescent poly(ethylene glycol) hydrogel microsensors. Sensors Actuators B Chem 105(2):365–377
32. Canal T, Peppas NA (1989) Correlation between mesh size and equilibrium degree of swelling of polymeric networks. J Biomed Mater Res 23:1183–1193
33. SAS Institute Inc. (2005) JMP Statistics and Graphics Guide. Cary, NC, pp 731–753
34. Shewhart W (1931) Economic control of quality of manufactured product. Van Nostrand, Princeton, NJ