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Glucose-Sensitive Holographic Sensors for Monitoring Bacterial Growth

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A glucose sensor comprising a reflection hologram incorporated into a thin, acrylamide hydrogel film bearing the *cis*-diol binding ligand, 3-acrylamidophenylboronic acid (3-APB), is described. The diffraction wavelength (color) of the hologram changes as the polymer swells upon binding *cis*-diols. The effect of various concentrations of glucose, a variety of mono- and disaccharides, and the α-hydroxy acid, lactate, on the holographic response was investigated. The sensor displayed reversible changes in diffraction wavelength as a function of *cis*-diol concentration, with the sensitivity of the system being dependent on the *cis*-diol tested. The effect of varying 3-APB concentration in the hydrogel on the holographic response to glucose was investigated, and maximum sensitivity was observed at a functional monomer concentration of 20 mol %. The potential for using this holographic sensor to detect real-time changes in bacterial cell metabolism was demonstrated by monitoring the germination and subsequent vegetative growth of *Bacillus subtilis* spores.

There is a requirement for sensor technologies that can be exploited to monitor cell metabolism in microbial fermentation processes and cell cultures. Such systems could be utilized in high-throughput screening to monitor the response of cells to potential therapeutics, optimize protein production, or monitor antibiotic or end product formation. The sensors may be used in small-volume microbial systems (nL) that can provide continuous measurements of metabolites of fermentation, particularly glucose,[1] and allow small-scale bioprocesses to be monitored, optimized, and controlled.

Conventionally, glucose sensors utilize the enzyme glucose oxidase; however, the use of catabolic enzymes is problematic because they consume their substrate and would significantly reduce substrate levels in small-volume bioreactors and thereby alter the metabolism of living cells. Furthermore, enzyme-based sensors often display poor stability as a result of long-term contact with process media [2] and problems associated with sterilization.[3]

Glucose levels in fermentation have also been monitored using high-performance liquid chromatography. However, this would not be appropriate for use in small-scale bioreactors as it requires destructive sampling.[4] Similarly, while near-infrared spectroscopy has also been utilized to monitor glucose levels in bacterial fermentations, this technique suffers from interference from other biological constituents and requires costly detection equipment.[5]

Thus, there is a clear need for stable, inexpensive, and "nonconsumptive" sensors for use at physiological pH values in bioprocess monitoring in small-volume bioreactors. Since synthetic recognition elements are generally more robust than their biological counterparts, and do not consume substrate by virtue of the fact they tend to be equilibrium rather than catalytic systems, they are particularly suited for use in monitoring small-volume biosystems. The most effective synthetic ligands for binding glucose in aqueous media are based on the use of boronic acids.[6]

The ability of boronic acid to reversibly bind *cis*-diols such as glucose has long been established.[7−10] At low pH, boronic acid exists in an uncharged, trigonal planar configuration (Figure 1a, **1**), which does not readily complex with *cis*-diols, although it can result in the formation of a strained molecule (Figure 1a, **3**),[11] which is easily hydrolyzed. The strained complex **3** can, however, react with OH^- to form the more stable tetrahedral state (Figure 1a, **4**), which possesses a negative charge.[12] At pH values above the pK_a, ∼8.8,[13] boronic acid exists in a tetrahedral state (Figure 1a, **2**) which binds *cis*-diols more readily.[13] Consequently, in most applications of these boronate analogues, the pH must be basic, i.e., >8, for effective binding of sugars. This presents a challenge for monitoring glucose in biological matrixes.

Thus, there has been a growing interest in the use of boronic acid ligands for detecting glucose in biological samples. Most of these detection systems have been based around the use of fluorescence,[14] which can suffer from the large background

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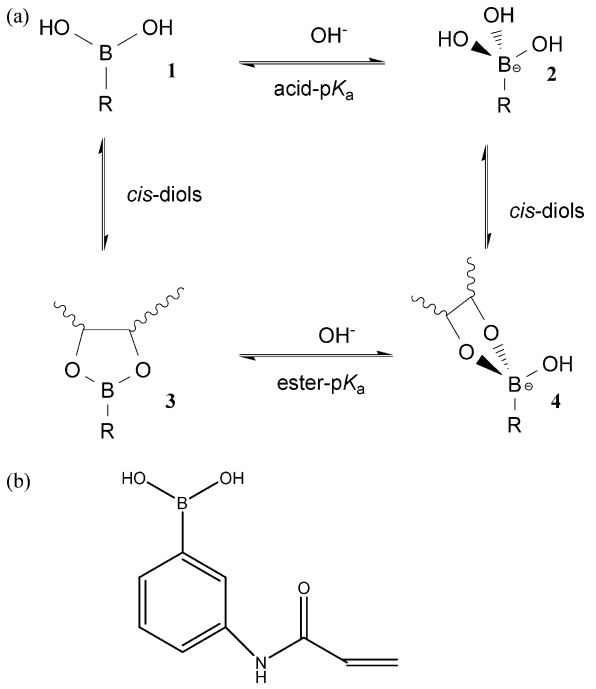

Figure 1. (a) Reversible binding that occurs between boronic acid and *cis*-diols in aqueous media. (b) Structure of 3-acrylamidophenylboronic acid.

fluorescence often found in complex biological media and may also be affected by temperature and oxygen concentration. Many fluorescence receptors often display low solubility in aqueous media, have low photochemical stability,[15] and may require the presence of solvents such as methanol to function.[16,17] Furthermore, the turbidity of cell growth media will also affect the performance of fluorescence-based optical sensors.[18] Electrochemical boronic acid-based glucose sensors have also been reported,[19,20] but these can suffer from surface fouling and interference from redox-active biological components.[21] A glucose sensor based on a boronic acid-containing hydrogel on a quartz crystal microbalance (QCM)[22] has also been reported. QCM transduction systems, however, are particularly difficult to implement in real-time and low-volume sensor systems.

A glucose sensor has been developed using phenylboronic acid groups copolymerized within an acrylamide hydrogel containing a colloidal crystalline array.[23] The colloidal crystalline array reports on the swelling state of the hydrogel by a change in the wavelength or color of diffracted light. At least one embodiment of this sensor functions at physiological pH and ionic strength, with the diffraction wavelength blue shifting at low glucose concentrations (up to 10 mM) and then red shifting at higher glucose concentrations. However, since the majority of these studies were performed at pH 8.5 and there is a change in direction of the diffraction wavelength shift at high glucose concentrations, this system may not be applicable to some bacterial fermentations, where the glucose concentrations can range from very high (>25 mM) to near zero. The fabrication of these sensors is also time-consuming and not easily amenable to mass manufacture.

An alternative method for detecting glucose using boronic acid ligands would be through the use of glucose-sensitive holographic sensors.[24] These sensors are based on planar small-volume polymer hydrogels containing silver halide, which acts as a holographic recording material. A reflection hologram is recorded within the polymer hydrogel matrix, producing an optical sensor that only reflects a narrow band of wavelengths when illuminated with white light.[25] The hydrogels used are termed "smart" polymers with ligands that interact with specific analytes incorporated into the polymer matrix.[25−32] This allows the hydrogel to respond to specific stimuli by changing its swelling state or hydration upon analyte binding.[33] The change in swelling state of the polymer causes a change in the wavelength of light diffracted by the holographic grating within the hydrogel. The narrow band of wavelengths diffracted is determined by the spacing between silver (Ag^0) nanoparticle fringes.[29] Thus, the hologram acts as a monochromatic mirror, in that light with a wavelength of approximately double the distance between the fringes will emerge in phase at a specific angle and constructive interference occurs. The diffraction wavelength of the hologram is governed by Bragg's law:

$$\lambda = 2nD \cos \theta$$

where θ is the angle of incidence, D is the distance between fringes, λ is the diffraction wavelength, and n is the average refractive index in vacuo.

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As the hologram swells, the diffraction wavelength increases and the color red shifts. Contraction of the polymer causes a decrease in diffracted wavelength and a blue shift. The changes in diffraction wavelength can be quantified using a suitable reflection spectrophotometer.

A holographic glucose sensor was produced initially by copolymerizing 4-vinylphenylboronic acid with acrylamide and *N*,*N*′-methylenebisacrylamide.[24] This sensor only responded well to glucose at pH 9 as the boronic acid ligand used was thought to have a high pK_a value.[20] It has been shown, however, that hydrogels made from 3-acrylamidophenylboronic acid (3-APB; Figure 1b) copolymers bind glucose at physiological pH.[12,34] Thus, copolymers of 3-APB and acrylamide with *N*,*N*′-methylenebisacryalmide as a cross-linker were synthesized and holograms recorded within them for use as glucose sensors. The response of the 3-APB copolymers to glucose, as well as a variety of saccharides, lactate, and a glycosylated protein, all of which contain *cis*-diols and can be found in cell growth media, was investigated. The effect of altering the mole percent of 3-APB in the hydrogel on the sensitivity to glucose was also studied. Finally, glucose depletion was monitored continuously as a function of bacterial growth using the holographic glucose sensor, to demonstrate utility of the new optical sensor under physiological conditions.

EXPERIMENTAL SECTION

Materials. All chemicals were of analytical grade unless otherwise stated. 1,1′-Diethyl-2,2′-cyanine iodide (QBS, photosensitizing dye), 2,2-dimethoxy-2-phenylacetophenone (DMPA), α_1-acid glycoprotein (AGP), acrylamide (electrophoresis grade), ammonium sulfate, β-deoxyribose, dimethyl sulfoxide (DMSO), dipotassium phosphate, β-D(−)-fructose, D(+)-galactose, hydrochloric acid, hydroquinone, L-lactate, lactose, magnesium sulfate, maltose, D(+)-mannose, monopotassium phosphate, *N*,*N*′-methylenebisacrylamide (MBA), potassium bromide, α-D(−)-ribose, silver nitrate (1 M, volumetric standard), sodium citrate, sodium hydroxide, sodium thiosulfate, sucrose, acryloyl chloride and tryptophan were purchased from Sigma Chemical Co.. Ascorbic acid and 3-aminophenylboronic acid monohydrate was obtained from Avocado Research Chemicals. D(+)-Glucose was purchased from ICN Biomedicals. 4-Methylaminophenol sulfate (Metol) was purchased from Acros Chemicals. Methanol and acetic acid (glacial) were obtained from Fisher Scientific Ltd. Phosphate-buffered saline (PBS) tablets providing 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl (pH 7.4 at 25°C) when dissolved in 200 mL of deionized water were purchased from Fluka. Tryptone soya broth was purchased from Oxoid Ltd. Bacto peptone digest was obtained from Difco. Thermo Electron Infinity Glucose Hexokinase kit purchased was from Thermo Trace (Melbourne, Australia).

Equipment. Microscope slides (Super Premium, 1−1.2 mm thick, low iron) were purchased from BDH (Merck) Ltd. Aluminized, 100-μm polyester film (grade MET401) was purchased from HiFi Industrial Film Ltd. A UV Exposure Unit (∼350 nm, model no. 555-279) was purchased from RS Components. A frequency-doubled Nd:YAG laser (350 mJ, 532 nm, Brilliant B, Quantel) was used in hologram construction.

Instrumentation. Holograms were analyzed using an Avantes AVS-MC2000-2 reflectance spectrometer and AvaSoft 5 processing software (Knight Scientific).

Synthesis of 3-Acrylamidophenylboronic Acid. A well-stirred, cooled solution of 3-aminophenylboronic acid monohydrate (5.0 g, 32.2 mmol) in aqueous sodium hydroxide (5.16 g, 129.04 mmol, 50 mL) was treated with dropwise addition of acryloyl chloride (5.2 mL, 64.4 mmol) over a period of ∼10 min. After 30 min of stirring on ice, the reaction mixture was allowed to reach room temperature and stirred for a further 2 h. The mixture was adjusted to pH 8 using dilute HCl (0.1 M). The resulting beige precipitate was filtered off and washed 5 times with 25 mL of cold water. Drying in air resulted in a fine powder, which was then dissolved in hot 20% (v/v) aqueous ethanol (∼50 mL) and filtered through fluted filter paper. The filtrate was left to stand overnight at room temperature, and the resulting crystals were filtered, crushed, and left to dry in a desiccator. The ^{1}H NMR spectrum of the product was consistent with previously published data,[34] and the purity of the product was >99% as adjudged by ^{1}H NMR integration.

Synthesis of Polymer Films. The appropriate quantity of each monomer was dissolved to give a prepolymer solution with the required molar ratios. In each case, the amount of cross-linker, MBA, was kept constant at 1.5 mol %. The monomers were dissolved in DMSO containing 2% (w/v) of the photoinitiator DMPA. The solid-to-solvent ratio of monomer to DMSO was kept constant throughout at 0.452:1 (w/v). A 100 μL droplet of monomer solution was pipetted onto the polyester surface of an aluminised polyester sheet sitting on a glass plate. A glass microscope slide, which had been treated with 3-(trimethoxysilyl)-propyl methacrylate,[31] was then gently lowered, silane-treated side down, onto the monomer mixture. Films were polymerized by UV-initiated free radical reaction at 20 °C for 30 min. Polymerized films were peeled off the polyester sheet while submerged in deionized water. Prior to hologram construction, the edges of each film were cleaned with a scalpel blade to remove any excess material.

Hologram Construction. Holograms were constructed using the diffusion method as described by Blyth et al.[35] The polymer films were placed face-down onto 400 μL of 0.2 M silver nitrate for 2 min. Excess solution was wiped off, and the film was dried in a stream of warm air. Under red safe lighting, the slides were placed film side up in 40 mL of 4% (w/v) KBr and 0.05% (w/v) ascorbic acid in 1:1 methanol/H_2O (v/v) with 1 mL of 0.1% (w/v) QBS in methanol and agitated for 1 min. The films were rinsed under distilled water and immersed polymer side down into the hologram exposure bath[31,26] containing PBS, pH 7.4, for 10 min. The whole area of the slide was then exposed to three single pulses from a frequency-doubled (532 nm) Nd:YAG laser. The slide was removed from the exposure bath and agitated for ∼10 s in freshly made developer solution (40 mL of 20 g/L ascorbic acid, 5 g/L 4-methylaminophenol sulfate, 20 g/L sodium carbonate, 6.5 g/L sodium hydroxide). The developed hologram was rinsed under distilled water and immersed in stop solution (5% (v/v) acetic acid). After rinsing under distilled water, the film was immersed for 5 min in agitated 20% (w/v) sodium thiosulfate to

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remove any undeveloped silver and rinsed in methanol and then distilled water.

Monitoring Holographic Responses. Single strips of hologram, ~8 mm wide were cut from a slide and inserted into a 4-mL plastic cuvette with the film side facing inward. PBS (1 mL) was added and the cuvette left overnight to equilibrate at 30 °C in a temperature-controlled cuvette holder. An Avantes AVS-MC2000-2 reflection spectrophotometer equipped with AvaSoft 5 processing software was used to measure the wavelength of light reflected from a white light source by the hologram as determined by spacing of the fringes within the polymer.[32]

The response of the holographic sensors to D(+)-glucose in PBS was tested. An overnight solution was used to ensure the relative proportion of glucose isomers present would mimic the equilibrium mixture found in media or biological fluids. The response of the sensor to the addition of six 20-μL aliquots of 0.1 M glucose in PBS to the cuvette was recorded. Between each addition, the sensor was allowed to equilibrate to a stable diffraction wavelength. A 2 × 5 mm magnetic follower (Fisher) and stirrer arrangement was used to ensure constant agitation.

Deoxyribose, fructose, galactose, lactate, lactose, maltose, mannose, ribose, and sucrose (0.1 M solutions) were also tested using a 12 mol % 3-APB hologram in a similar manner.

The heavily glycosylated protein (45 wt %), AGP, was selected as a model glycoprotein. It has a molecular mass of 41−43 kDa, and 45% of its weight is due to its glycosylated chains.[36] The response of the hologram to ≤3 mg/mL AGP was investigated.

Monitoring Glucose Depletion in a Culture Medium. A 12 mol % 3-APB hologram was equilibrated in 2 mL of fresh minimal medium. The medium consisted of 7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 0.5 g/L $C_6H_7NaO_7$, 0.1 g/L MgSO4, 1 g/L $(NH4)_2SO_4$, 5 g/L glucose, and 5 g/L Bacto peptone digest at pH 7.1. *Bacillus subtilis* NCTC3610 spores were prepared using the CCY medium method as described by Stewart et al.[37] and stored in water at 4 °C. A 4-μL aliquot of spores was added to the medium containing the hologram to give a final concentration of 4.2×10^7 cells/mL (as determined by plate counts).

The cuvette was incubated at 30 °C, agitated using a magnetic stirrer, and the holographic diffraction wavelength recorded for 10 h. The cuvette was capped with a sterile sponge bung to allow free diffusion of oxygen. The medium was aerated using a peristaltic pump with a 0.2-μm Minisart (Sartorius) filter attached to a sterile needle. To verify the glucose concentration of the medium, 50-μL samples were removed from the cuvette at specific time points using a sterile syringe. The samples were centrifuged for 1 min at 16000*g*, and the supernatant was frozen using liquid nitrogen. Glucose determination of the supernatant was performed spectrophometrically using a Thermo Electron Infinity Glucose Hexokinase kit (Catalog No. TR15421). Glucose concentrations in the growth medium were interpolated from a calibration curve comprising culture medium supplemented with 0−25 mM glucose by reference to a best-fit linear equation. Triplicate measurements were typically <±5% for glucose measurements with the hexokinase kit. The optical density (*A* 600 nm) of the growing bacteria was also monitored in identical experimental conditions using a HP 8452A diode array spectrophotometer. The configuration of the hologram in the cuvette precluded any affect of the cell density on the diffraction wavelength since the optical path for the reflection hologram did not traverse the cell suspension. The pH of the medium was also monitored using a Hanna microelectrode.

RESULTS AND DISCUSSION

The response of a 12 mol % 3-APB hologram to glucose in PBS pH 7.4 at 30°C was investigated. Figure 2a shows that the diffraction wavelength of the hologram red shifts on addition of glucose, indicating that the polymer expands upon binding glucose. A similar result was observed by Asher et al.[38] with an acrylamide copolymer containing pendant phenylboronic acid groups. They attributed this to the formation of charged, tetrahedral phenylboronate groups (Figure 1a, **4**) on binding of glucose. The presence of the charged boronate anions creates a Donnan potential, which results in water partitioning into the hydrogel and thereby causing it to swell. The wavelength response of the sensor was directly related to the glucose concentration (Figure 2b) over the normal physiological range of blood glucose concentrations in humans[39] and could be extended up to 150 mM. The half time ($t_{1/2}$) required to reach the maximum diffraction wavelength change at 2 mM glucose in PBS buffer, pH 7.4, with a 12 mol % 3-APB hologram was 10.5 ± 0.99 min for triplicate measurements. The calibration of the sensor deviated by 10% or less for variations in ionic strength of up to 50 mM NaCl in PBS buffer, pH 7.4, indicating a low susceptibility to changes in ionic strength within the range normally anticipated in a fermentation broth.

When the hologram was thoroughly washed in an excess of buffer, the peak returned to within ±3 nm of the initial baseline wavelength, demonstrating the fully reversible response of the sensor. The sensor was stored dry at room temperature and could be used repeatedly over the course of several months without significant degradation of the response to glucose, attesting to the robust nature of holographic sensors.[26]

A control copolymer comprising 12 mol % *N*-phenylacrylamide instead of 3-APB in acrylamide produced a sensor that displayed no response to glucose, indicating that the observed shifts in diffraction wavelength with 3-APB copolymers were due to the interaction of glucose with the phenylboronic acid ligand, rather than nonspecific interaction with the polymer backbone.

The sensitivity of holographic sensors can be controlled by altering the amount of the active monomer or ligand present in the hydrogel, with the sensitivity of the system generally increasing with active monomer concentration.[26,32] Thus, the mole percent of 3-APB incorporated into the acrylamide copolymer was varied and the response to glucose tested in PBS (Figure 3).

Sensitivity to glucose increased as a function of mole percent of 3-APB in the polymer up to 20 mol % (Figure 3a). Using a 20 mol % 3-APB hologram, a change of ~0.5 mM glucose could be resolved, based on the resolution of the detection system used. The small error bars in Figure 3a demonstrate the reproducibility of the sensor. Furthermore, as seen in Figure 3b, a maximum

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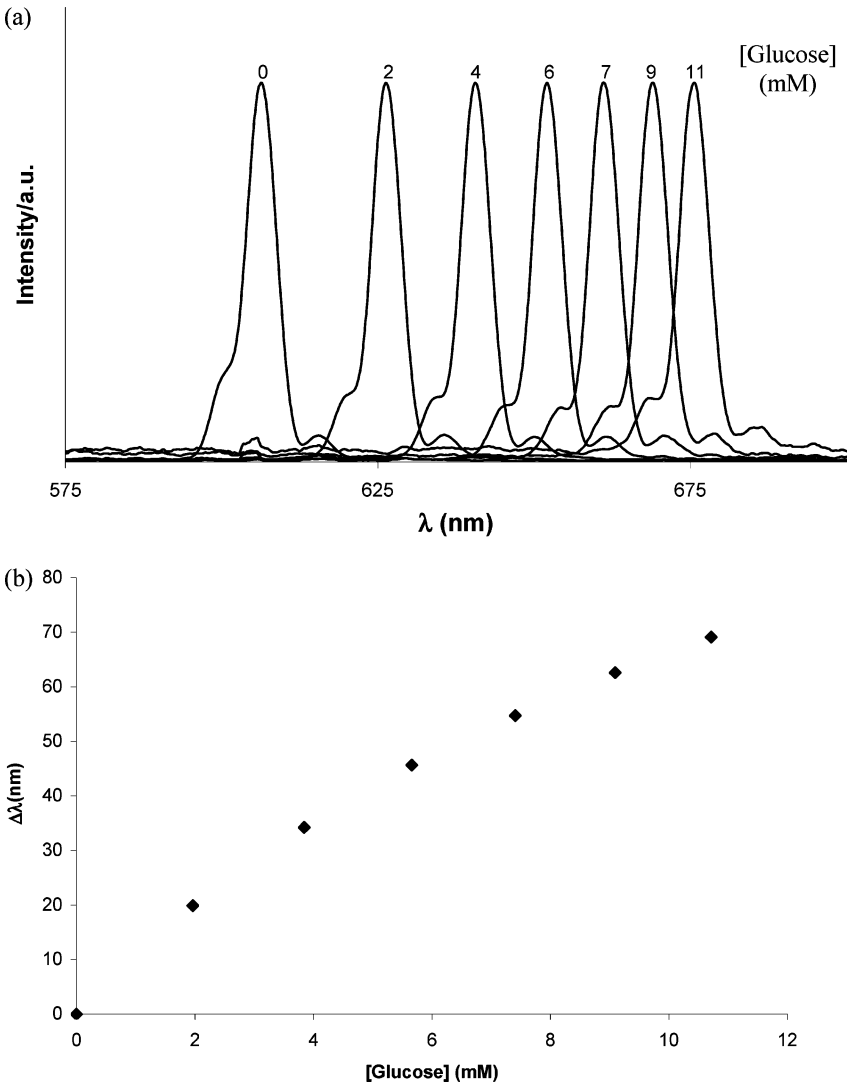

Figure 2. (a) Change in diffraction wavelength of a hologram containing 12 mol % 3-APB as a function of glucose concentration in PBS pH 7.4 at 30 °C. The glucose concentration values are displayed above the diffraction peaks. The hologram red shifts with an increase in glucose concentration and returns to within 3 nm of the original diffraction wavelength when washed with PBS. (b) Calibration curve for diffraction wavelength versus glucose concentration.

response toward glucose was observed at 20 mol % 3-APB. Above this concentration, the sensor became less responsive, presumably due to alterations in the elastic properties of the polymer at high ligand concentrations, rendering it less sensitive to modulation by glucose binding.[32] Since 3-APB is a planar, hydrophobic molecule, it is possible that the polymer simply becomes more rigid as 3-APB molecules stack together within the polymer at high mol percentages.

Boronic acids are known to bind a wide variety of *cis*-diols.[7,16,40] As such, any sensor system utilizing boronic acid ligands would be susceptible to interference from competing *cis*-diol-containing compounds in the sensing environment. The sensor response to various mono- and disaccharides was tested in order to investigate the cross-reactivity of the holographic glucose sensor with other analytes commonly found in cell culture media (Figure 4). The response to lactate, an α-hydroxy acid, which is an important product of anaerobic metabolism, was also investigated as it has been shown that lactate can bind to boronic acid.[40]

The position of the hydroxyl groups on different sugars, such as mannose and galactose, affects the affinity and thus the response of the sensor when compared to glucose, as binding is known to occur only with suitably configured *cis*-diols.[41] The hologram responded fastest to fructose, which also caused the greatest swelling and wavelength change of the sensor. Similarly, ribose gave a large response, suggesting that five-membered furanose rings offer the most suitable positioning of diols for binding to boronic acid. It has been suggested by van den Berg and van Bekkum[42] that the *cis*-1,2 diol configuration on the furanose ring produces a very stable borate ester with a low ring strain and a decrease in entropy of the ligand. Deoxyribose displayed little diffraction wavelength shift compared to ribose, indicating the importance of the hydroxyl group on carbon 2, which is present in ribose but absent in deoxyribose (Figure 4). The disaccharides, lactose, maltose, and sucrose, showed poor response, probably due to steric hindrance caused by the glyco-

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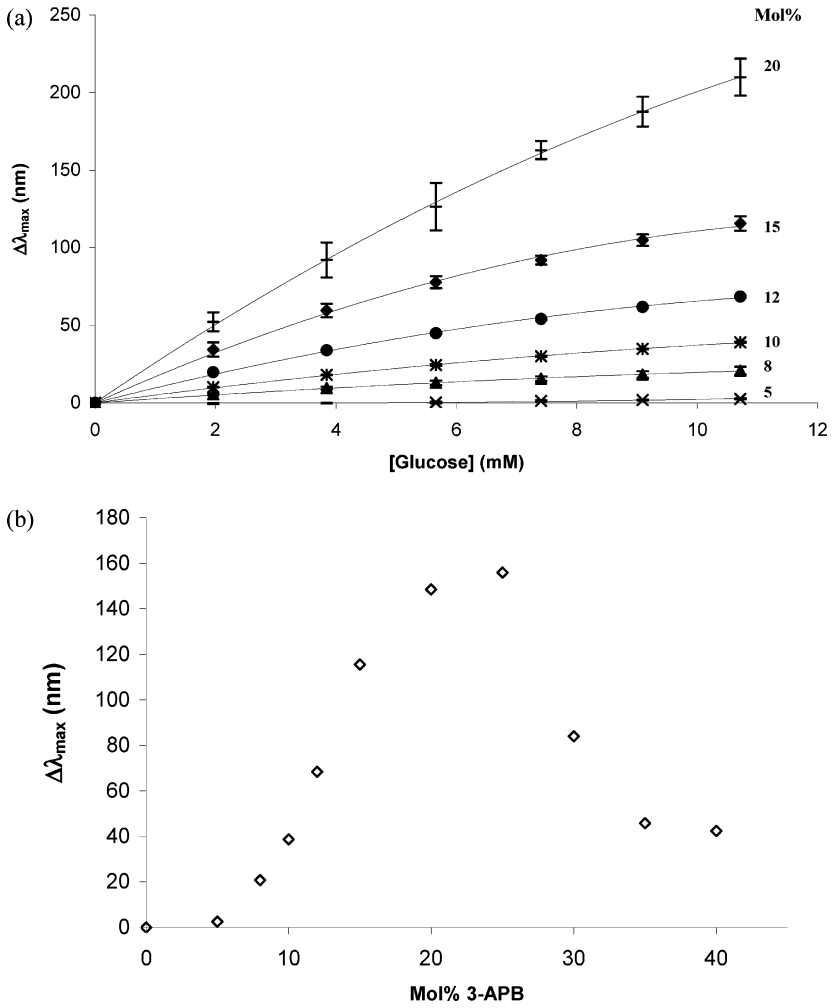

Figure 3. (a) Response of holograms containing 5, 8, 10, 12, 15, and 20 mol % 3-APB to glucose in PBS pH 7.4 at 30 °C. Error bars represent ±1 standard deviation ($n = 3$). (b) Change in diffraction wavelength shift as a function of mol % 3-APB with 10.7 mM glucose in PBS pH 7.4 at 30 °C.

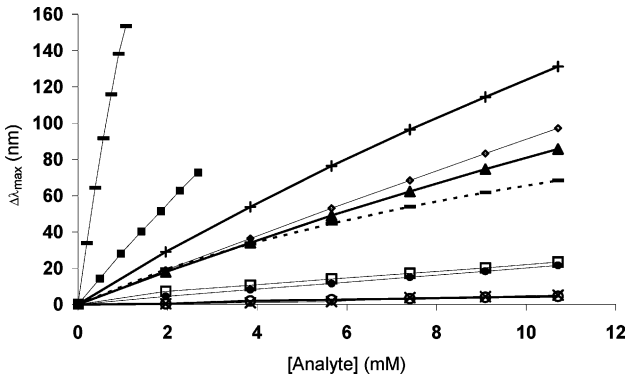

Figure 4. Response of a 12 mol % 3-APB hologram to various saccharides and lactate in PBS pH 7.4 at 30 °C. Key: —, fructose; ■, ribose; +, galactose; ◇, lactate; ▲, mannose; - - -, glucose; □, lactose; ●, maltose; ○, sucrose; ×, deoxyribose.

sidic bond and the fact that certain diol groups were no longer available for binding. The order of binding affinities deduced from the magnitudes of the holographic response to various analytes agrees with the data obtained by Springsteen and Wang,[11] using a competitive fluorescence assay. These results highlight the relatively low selectivity of the sensor, but with mammalian and many microbial cell cultures, this would not be a significant issue as the main carbon source in growth media is glucose.

The sensor was found to respond to the α-hydroxy acid, lactate, as it also possesses a suitable *cis*-diol structure.[43] The system responded faster to lactate ($t_{1/2}$ 0.7 min; 2 mM lactate) than glucose ($t_{1/2}$ 10.5 min; 2 mM glucose), possibly due to flexible orientation of *cis*-diols in lactate and its small size, which would allow it to diffuse more rapidly into the polymer than the larger glucose molecule. To investigate whether lactate binds to 3-APB using its carboxylic acid group only or using its two *cis*-diols, a *β*-hydroxy acid, 3-hydroxybutyric acid, which possesses a carboxylic acid group and a *β*-hydroxyl group but no *cis*-diols, was tested. The sensor showed no response to 3-hydroxybutyric acid, suggesting that binding of lactate occurs through the *cis*-diols present and not just the carboxylic acid group. A similar result was observed with the monocarboxylic acid acetic acid.

Although the holographic glucose sensor does respond to a variety of monosaccharides, in most cases, these will not be found in abundance in growth media unless they are used as the primary carbon source. However, saccharides are present on the surface of cells and on proteins, some of which may be produced and

(43) Gray, C. W., Jr.; Houston, T. A. *J. Org. Chem.* **2002**, *67*, 5426−5428.

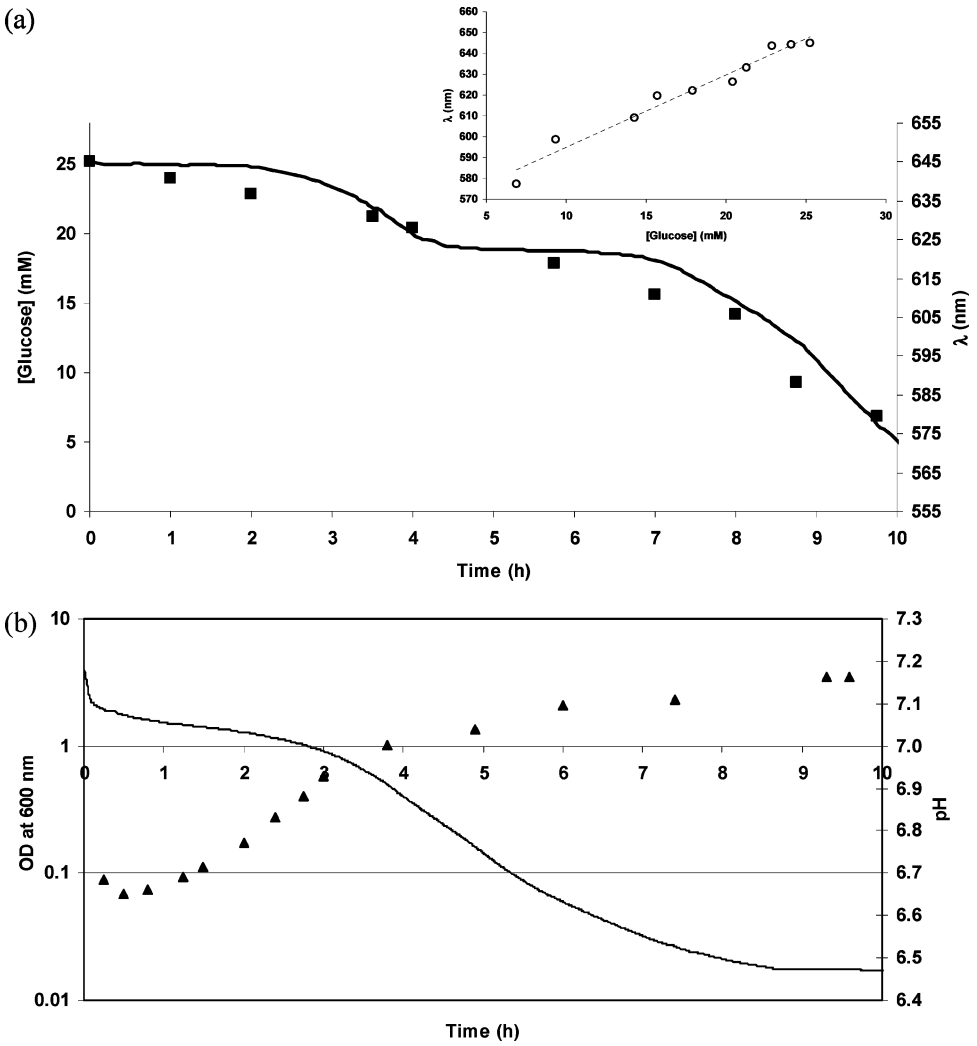

Figure 5. (a) Change in diffraction wavelength of a 12 mol % 3-APB hologram in the presence of growing *B. subtilis* and decreasing glucose levels determined by an enzymatic hexokinase method; ■, hexokinase; —, hologram. The inset shows the correlation between real glucose concentrations and diffraction wavelength at specific time points during growth. (b) Optical density and pH of the growing spores under the same conditions; ▲, OD; —, pH.

secreted by the cells, as glycosylated products.[44] These structures could potentially interfere with the operation of the sensor as they would be able to bind to 3-APB. Thus, the response of the sensor to a model glycoprotein, α_1-acid glycoprotein, was assessed to see if it could interfere with glucose detection.

AGP is an acute phase protein present at a concentration of 0.2−1 mg/mL in normal human blood, which increases by 2−3 times during inflammation.[45] The sensor displayed no response to AGP in PBS. This was probably due to the large size of the protein, which was unable to penetrate significantly into the cross-linked polymer matrix. Thus, glycosylated proteins, whether present as part of a complex growth medium, or excreted during cell growth, should not affect the response of the sensor. In addition, glycosylated structures, which are normally present on the surfaces of cells, would not be expected to interfere with glucose detection.

The depletion of glucose during the growth of a model bacterial system was monitored in order to prove the feasibility of using this sensor to monitor fermentation processes. Previously, holographic sensors were successfully used to monitor the homolactic fermentation of milk by *Lactobacillus casei* through the change in pH generated during the fermentation process.[26] In the present report, the growth of *B. subtilis* was chosen as a model system for cell growth, since this particular strain is simple to grow in a reproducible fashion under well-defined conditions. *B. subtilis* is an obligate aerobe; therefore, products of anaerobic fermentation such as lactate, which may interfere with the sensor, are unlikely to be produced in significant quantities.

Figure 5a shows a gradual decrease in the diffraction wavelength of the hologram after addition of bacterial spores, suggesting a depletion of glucose in the medium. The determination of glucose with the independent hexokinase method suggested that the decrease in diffraction wavelength observed was due to decreasing glucose concentrations. Bacterial growth was confirmed by monitoring the increase in optical density at 600 nm of a separate experiment performed under identical conditions, in the presence of a hologram; the population was observed to enter stationary phase after 10 h (Figure 5b). With an OD $\approx$ 1, the cell density was found to be 1.8×10^8 cfu/mL as determined by plate

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(45) Shiyan, S. D.; Bovin, N. V. *Glycoconjugate J.* **1997**, *14*, 631−638.

counts. Figure 5a also shows that the trend observed with the diffraction wavelength shift mirrored that of the external glucose determination method. The inset in Figure 5a suggested a linear correlation between the measured glucose concentrations and diffraction wavelength values during the growth period.

It should be possible to correlate the diffraction wavelength to actual glucose concentrations by use of a calibration curve, which would provide the diffraction wavelength for known concentrations of glucose in the growth media. However, the pH of the culture medium was found to decrease during spore germination and growth (Figure 5b). The hydrogel contracts as the pH decreases[26] since 3-APB is an acid with a pK_a of ~8.6.[46] Furthermore, as seen in Figure 1a, the sensitivity of boronic acids toward *cis*-diols decreases with pH.[8] Both of these factors would alter the overall response to glucose of the holographic glucose sensor as a function of pH. These two factors may be the cause of the change in the diffraction wavelength seen at ~4 h in Figure 5a. Thus, it would be necessary to calibrate the sensor for changes in pH over the glucose concentration range to convert the diffraction wavelength observed directly into a true glucose concentration. However, despite this response of the sensor to changes in pH, there is a clear correlation between the holographic sensor response and glucose depletion (Figure 5a).

One of the key advantages of the holographic sensor system is that it is possible to produce multiplexed sensor systems with an array of different holograms for different analytes. Thus, a suitable pH hologram, such as that described by Marshall et al.,[26] could be used in tandem with the glucose sensor to record the pH of the medium continuously. The pH data could then be used to correct the response of the sensor for the changes in pH during cell growth. A suitable lactate sensor could also be used in tandem to quantitate lactate in anaerobic fermentations and correct for responses to lactate. Ultimately, the system would operate through an array of holographic sensors, to detect a variety of parameters associated with cell metabolism, for use in monitoring cell growth and fermentation processes. The array could be customized to accommodate selected analytes associated with specific cell growth processes.

CONCLUSIONS

A reversible, real-time holographic sensor for glucose was fabricated, using 3-APB as a glucose binding ligand. Although the sensor does not display unique specificity for glucose, glucose is normally the predominant free sugar used in the majority of cell growth media as the primary carbon source. The sensor does not respond to macromolecular glycosylated structures, such as glycoproteins, which cannot diffuse into the hydrogel matrix. Characterization of the sensor revealed a high affinity for furanose ring structures, such as those of fructose and ribose, and an affinity for the α-hydroxy acid, lactate, which maybe problematic as it is a product of anaerobic cell metabolism. Preliminary studies using a live aerobic culture of *B. subtilis* spores demonstrated that the sensor could be used to monitor glucose depletion in the culture media with a good correlation between the sensor response and glucose concentration.

Unlike enzyme-based glucose sensors, the holographic sensor is nonconsumptive, amenable to mass manufacture, and easily miniaturizable, which makes it suitable for use in small-volume microfermentor systems. The absence of a biological recognition system also makes it more robust, stable, and less expensive to manufacture. The sensor can be read using a simple reflection spectrometer and does not require large, dedicated pieces of equipment. Although the current sensor displays low selectivity and is affected by pH, these variables could be measured through the use of other holograms in an array and compensated for by use of suitable algorithms.

Forthcoming studies will focus on improving both the selectivity and response kinetics of the sensor. Modification of the ligand, the polymer hydrogel, or both could be used to reduce or eliminate interference and improve the kinetics of the sensor. The sensor will also be tested on a wider range of cell fermentations, including other bacterial and mammalian cells, and in small-volume systems. Furthermore, the sensor should be applicable to blood glucose monitoring since it displays a progressive response to glucose concentrations up to at least 25 mM at physiological pH (7.4) and is only minimally affected by ionic strength at this pH. It is anticipated that the sensor will function in whole blood.

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