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A REAL TIME OPTICAL BIOSENSOR ASSAY FOR AMOXICILLIN AND OTHER β-LACTAMS IN WATER SAMPLES

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Short title: Biolayer interferometry assay for amoxicillin

ABSTRACT

Antibiotic contamination of drinking water and sewage is a matter of environmental and public health concern. Traditionally, ELISA or HPLC methods have been used to detect and measure antibiotic contamination. By applying an optical biosensing method, biolayer inteferometry (BLI), we have developed a kinetic competition binding assay capable of quantitating less than 1ppm (~33 µM) amoxicillin. Similar to surface plasmon resonance, BLI senses changes that occur upon binding of one molecule to another near a surface to measure association and dissociation. Immobilized amoxicillin was used to screen for binding against an analyte solution of anti-amoxicillin equilibrated with amoxicillin-containing water samples, yielding binding that fit a one-state model. Maximal binding correlated highly with amoxicillin concentration. Simplified analysis of samples from water and sewage treatment plants in Georgia allowed quantitation without kinetic modeling. The assay is sensitive, cost-effective, fast and readily adaptable to a variety of samples and other small molecules.

Keywords: biosensor, biolayer interferometry, amoxicillin, β-lactam contamination
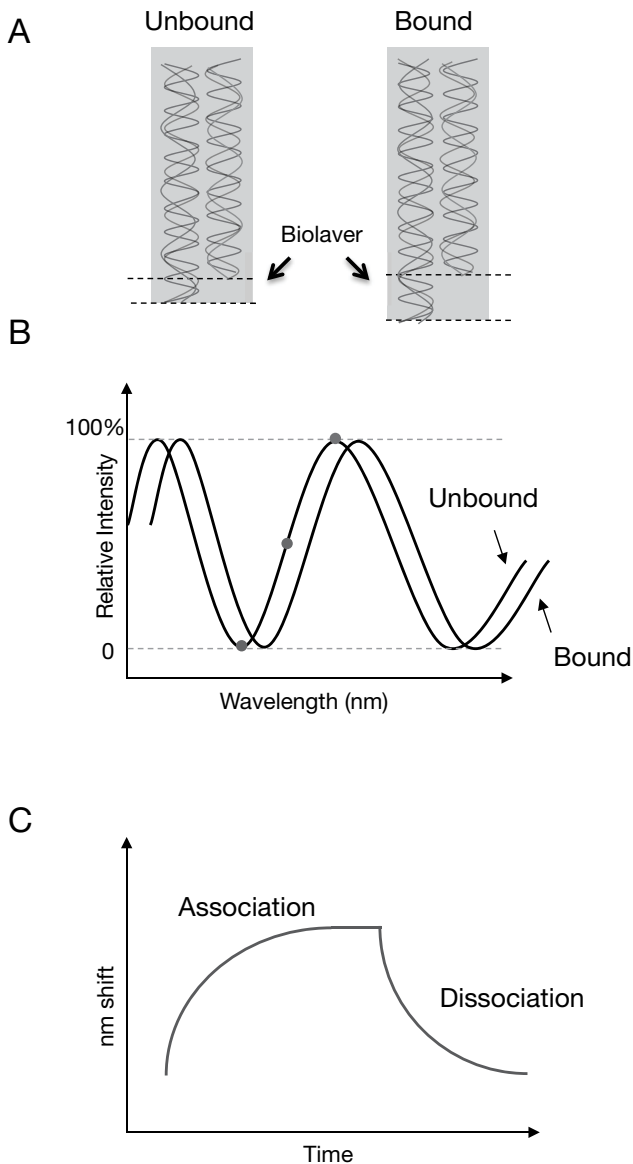
INTRODUCTION

Environmental antimicrobial contamination is a subject of concern (1), chiefly that overuse of antibiotics leads to increases in resistant microbes. Aquatic environments are areas of special concern, since medical, agricultural

and other sources of antibiotic pollution accumulate in waste water and the effects are not well understood (2, 3).

β-lactams, the class to which amoxicillin belongs, are among the most commonly prescribed antibiotics. Standard methods for measuring amoxicillin and other antibiotics generally involve chromatographic separation followed by spectrophotometric quantitation (4, 5). Limits of detection (LOD) range from ~0.1-1 µg ml^{-1}, with the limits of quantitation (LOQ) being somewhat higher. More exotic methods have been developed, including chemilumenescent flow injection which allows greater sensitivity (10 pg ml^{-1}) but requires complex set-up and handling procedures (6). Immunoassays such as competitive ELISAs have demonstrated excellent agreement with HPLC procedures at higher concentrations with greater sensitivities allowing for accurate measurements to 10 ng ml^{-1} (7, 8). The higher sensitivity is somewhat mitigated by long incubation times that require hours to collect data.

In contrast, the assay described herein is both sensitive and rapid. Optical biosensing is a technology that allows for real-time, label-free kinetic analysis and quantitation of biomolecules. A biosensor assay for quantitation of β-lactams with a novel but somewhat complex use of penicillin binding protein has been described (9). That study used a surface plasmon resonance (SPR) instrument that employs very expensive consumables and easily fouled microfluidic components. Our biolayer interferometry (BLI) assay uses a simpler strategy, cheaper consumables and a commercially available antibody.

The ForteBio Octet system used in this study is a commercial BLI biosensor (Fig. 1). The BLI principle shares some similarities with SPR in that changes measured near a surface reflect association and dissociation of biomolecules from which affinity and kinetic information can be determined. Briefly, one molecule, the ligand, is immobilized to a proprietary "biolayer" at the tip of fiber optic sensors by one of several methods; this study utilized covalently immobilized bovine serum albumin (BSA)-conjugated amoxicillin as ligand. White light passing down the fiber optic can reflect off of an internal reference or the biolayer. The resulting interference pattern of the reflected light is measured by a photodetector. After a baseline reading, sensors are dipped into buffer containing the binding partner, or analyte. Analyte binding to ligand results in an increase in distance between the reflecting surfaces and a coincident change in the interference pattern. Changes in the interference pattern are reported via signal processing as "nm shift." After association, sensors are moved to buffer not containing analyte and dissociation is measured as the interference shift moves back toward the unbound pattern. Rate and affinity constants can be determined by fitting association and dissociation response curves. The instrument operates in an 8 x 12, 96-well format allowing for up to eight simultaneous experiments with different analyte concentrations, serial repetition and automated operation. Other advantages include the ability to recover samples and an absence of microfluidics, which allows for use of crude samples containing particulates.

A

Unbound   Bound

Biolayer

B

100%

Relative Intensity

Unbound

0

Bound

Wavelength (nm)

C

Association

nm shift

Dissociation

Time

Figure 1. The biolayer interferometry principle. A, an interference pattern is established by reflection of white light from two surfaces (dashed lines) on a fiber optic sensor with biotinylated molecule attached to the biolayer at the tip of the optic. The length light must travel to reflect off of the second surface increases upon analyte binding due to an increase in the optical thickness of the biolayer. B, binding of analyte molecule causes a shift in the wavelength of the interference pattern. C, monitoring of interference shift over time reflects binding of analyte to the immobilized molecule. Association

is measured in the presence of analyte. Dissociation is measured when the sensor is moved to buffer not containing analyte. This figure is modified and used with permission from Dr. Krista Witte (ForteBio).

A competition binding strategy was employed. Sensor-bound amoxicillin was exposed to solutions of free amoxicillin or environmental water samples equilibrated with anti-amoxicillin antibodies. Response varied inversely with free amoxicillin concentration with first order kinetics dependent on free antibody concentration. Similar strategies have long been applied to small molecule and other assays using saturation binding of radioligands, ELISA, and other techniques.

A limiting factor in the use of biosensors for small molecule assays is cost. Consumable sensors represent most of the per assay cost, though other reagents can be significant. Efforts were made to optimize antibody concentration to allow determination of free amoxicillin concentration within the desired range without waste of antibody. Methods to regenerate sensors for repeated use were also explored.

MATERIALS & METHODS

All BLI measurements were made on a ForteBio (Menlo Park, CA) Octet QK biosensor using screening grade Amine Reactive Biosensors (equivalent sensors are now sold as "AR Sensors"). Amoxicillin was purchased from Sigma-Aldrich. Unconjugated monoclonal mouse anti-amoxicillin and BSA-conjugated amoxicillin (BSA-AMOX) were purchased from US Biological (Swampscott, MA).

Development of assay for amoxicillin concentration. Assays were performed in 96-well microplates, a typical setup for which is shown in Table I. All volumes were 200 µl. Measurements were made at 25°C. Typically, sensors were first activated by dipping into a mixture of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS) for 600 s. Sensors were then moved to BSA-AMOX diluted from stock 1:100 (~40 ng/ml final) in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 5.0 for 900 s. Crosslinking was quenched in 1 M ethanolamine, pH 8.5 for 900s. Baseline measurements were made in buffer TBST (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 900 s. In assay development experiments, sensors were then exposed to anti-amoxicillin (a 1:500 final dilution in TBST from stock) equilibrated with various concentrations of free amoxicillin diluted in TBST ranging from 0-100 µM. Association response was monitored for 900 s after which the sensors were moved to TBST only for monitoring of dissociation for 900 s. Reference experiments in which conditions were identical save no ligand was present were performed to assure that no response was observed in the absence of ligand. Those experiments yielded only baseline response, making continuing reference subtraction unnecessary, *i.e.* nonspecific binding was negligible.

Table I. Plate setup for typical experiment. Rows A-H differed only in the concentration of free amoxicillin in association phase (column 5). The instrument is able to dip a sensor in each well of a column simultaneously, ensuring identical exposure to loading, quenching, etc.

	Activation	Loading	Quenching	Baseline	Association	Dissociation
	1	2	3	4	5	6
A	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + buffer only	TBST
B	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #1	TBST
C	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #2	TBST
D	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #3	TBST
E	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #4	TBST
F	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #5	TBST
G	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #6	TBST
H	EDC/NHS	BSA-AMOX	Ethanolamine	TBST	Ab + [AMOX] #7	TBST

Assay of environmental samples. Water samples were collected from inlets and outlets at the Newton County, GA Waste Water Treatment Facility and Water Resources Plant at Cornish Creek. Due to time between collection and assay development, samples were lyophilized, frozen and the resuspended to their original volumes in TBST prior to assay. Competition binding experiments were performed as above except that baseline measurements were made in diluted samples in the absence of antibody. Qualitative yes/no assays for presence of amoxicillin were performed followed by tuning experiments for appropriate dilution of the sample in TBST to reach a concentration within the range of sensitivity determined during development.

Modeling. Association responses were fit to a one state model using GraphPad Prism 5.0 (GraphPad Software, La Jolla CA), the equation for which was $Y = Y_0 + A(1-e^{(-kobs*t)})$ where Y = nm shift, Y_0 = initial shift at start

of the association phase, A = amplitude, *i.e.* the difference between maximal and minimal binding, and k_{obs} is the observed time constant (s^{-1}).

A competition binding curve was fit using Y = Bottom + ((Top-Bottom)/$1+10^{x-logEC50}$) where Y = percent of maximal shift (100% defined as response in the absence of competing amoxicillin) and Top and Bottom were the upper and lower plateaus, respectively. Response was expressed as percent maximal response with amplitude in the absence of competing amoxicillin defined as 100%.

Simplified determination of the concentration of amoxicillin in environmental samples was performed using linear regression of shift value at 900 s versus concentration of amoxicillin using a range of standards that evinced a linear response.

Regeneration. Sensors were used once for assay development. However, in order to enhance the cost-effectiveness, regeneration of sensors for repeated rounds of binding was investigated using a number of approaches. The best regeneration occurred when sensors were dipped into 10 mM glycine, pH 2.0 for 30 s followed by dipping into TBST for 30 s. That cycle was repeated for a total of three times prior to reuse.

RESULTS

Assay development. The raw ouput of a typical competition binding experiment is shown in Fig. 2. Activation, loading and quenching yielded sensors charged BSA-AMOX. After baseline measurement, association was performed in the presence of constant antibody and various concentrations of free amoxicillin. Response varied inversely with [amoxicillin], *i.e.*, the more amoxicillin in solution, the less antibody was available for binding to sensor-bound amoxicillin. Dissociation phases demonstrated typically negligible dissociation of antibody from antigen.
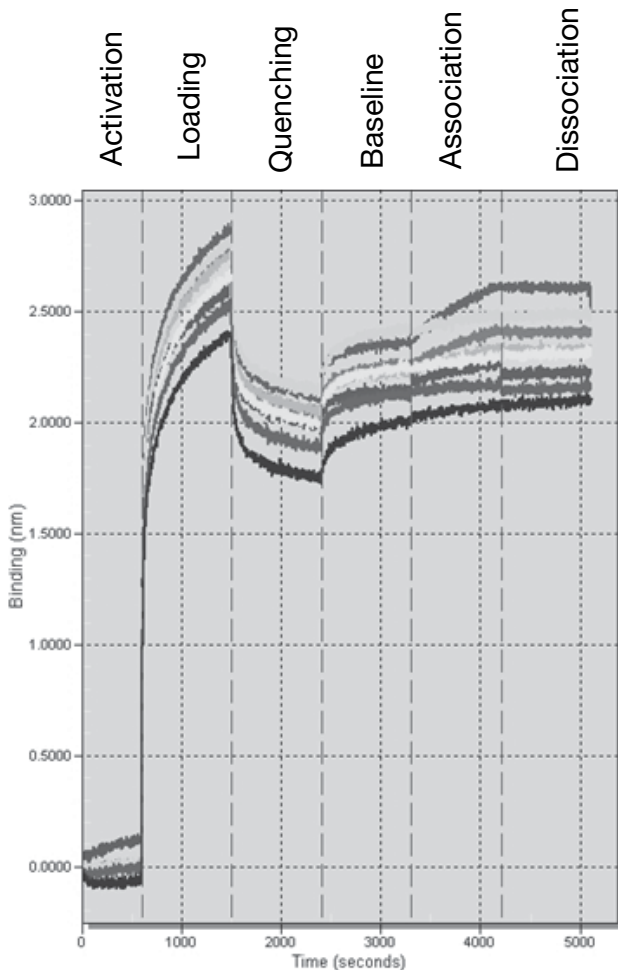
Figure 2. Course of a BLI experiment. Sensors were initially dipped into EDC/NHS to activate the crosslinking reaction. BSA-AMOX was loaded in the second phase and then quenched in 1 M ethanolamine, pH 8.5 in the third. After baseline in TBST, sensors were dipped into solutions containing amoxicillin and anti-amoxicillin and association was measured. Dissociation was observed upon movement of sensors into TBST without antibodies.

Parameters were determined empirically with consideration for both cost and sensitivity. Dilution of the BSA-AMOX ligand from stock 1:100 gave maximal response against no amoxicillin, *i.e.* no additional response was seen versus higher concentrations of BSA-AMOX in the loading phase (data not shown). Other parameters, *e.g.* extended quenching times, made no difference in response. For data analysis, only association phase shifts were used, with the shift at the beginning of the phase defined as zero.

Various dilutions of antibody were attempted, resulting in lack of sensitivity in the desired range (0- ~30 μM), with more antibody yielding sensitivity at higher concentrations of amoxicillin and less antibody sensitive at lower concentrations with a commensurate drop in overall response. A final antibody dilution of 1:500 from the stock solution was employed and gave sensitive responses between 0 and 10 μM.

The 1:500 dilution was chosen as a compromise among signal, sensitivity and cost. Lower concentrations of antibody yielded lower maximal responses, often to the degree that differentiation among responses was immeasurable. Higher concentrations of antibody that could result in accurate quantitation of higher concentrations were deemed too expensive, forcing dilution of samples to achieve sensitivity.

A range of concentrations of free amoxicillin from 0 to 20 μM was tested with 1:500 antibody. Raw data from association phases with fits to a one-phase association model are shown in Fig. 3A. Response plateaus, i.e. maximum shift values for each concentration, were determined from modeling. Percent maximal shift (max shift defined as the plateau obtained in the absence of competing amoxicillin) versus log [amoxicillin] yielded a competition binding curve for which $R^2 = 0.98$. (Fig. 3B). EC_{50} was 1.1 μM. Near-maximal inhibition of response was seen with samples containing over 5 μM amoxicillin.

A

B
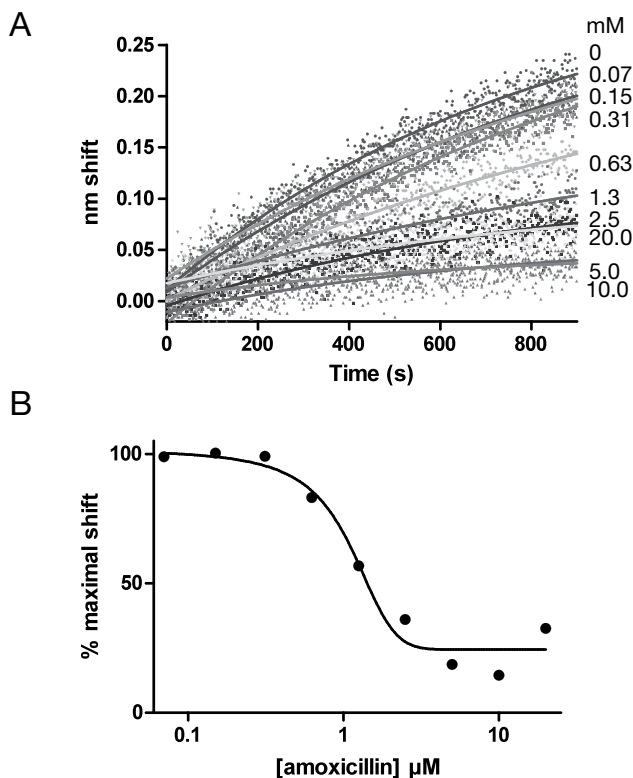
Figure 3. Association response varies as a function of amoxicillin concentration. A, association phase of competition experiments with amoxicillin concentrations ranging from 0-20 μM. Concentrations are shown at right. Association response was fit to a one-state binding model shown as solid lines among the raw data. Data are from two separate experiments performed with the same reagents. B, competition binding curve with response expressed as % maximal binding garnered from determination of the plateau response from A.

A second competition experiment was performed using multiple samples within the range sensitivity of the first competition binding curve. That experiment showed a substantial difference from the no amoxicillin control at concentrations as low as 0.2 μM (Fig. 4A). Maximal response was linear up to 2.4 μM (R^2=0.96)(Fig. 4B). The 1.6 μM sample was dismissed because of ambiguous determination of its plateau. The high degree of linearity among the 0.2-2.4 μM samples defined the range into which environmental samples to be measured must fall.
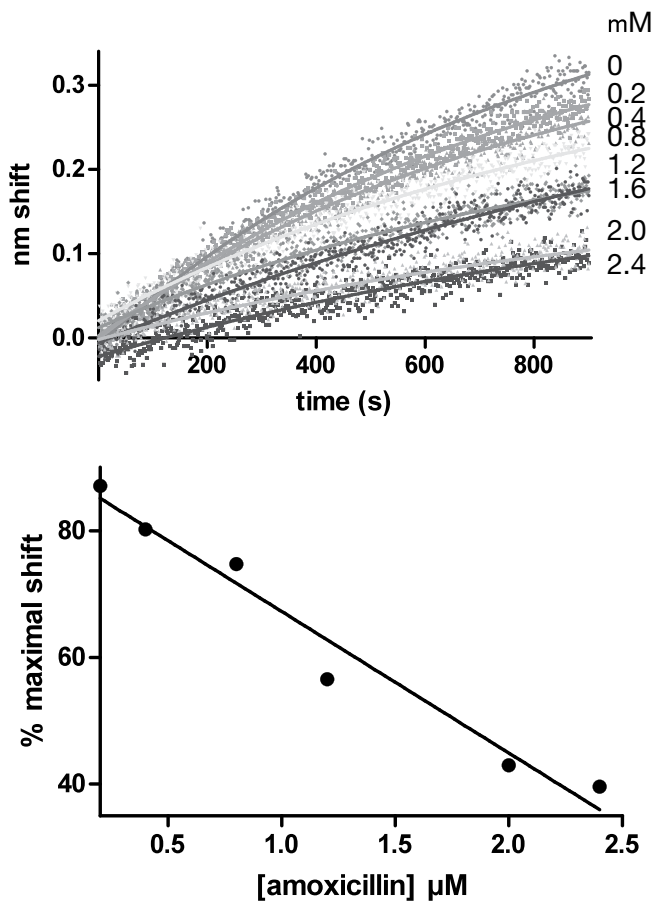
Figure 4. Linear response range of assay. Competition association experiment over a narrow concentration range effects a linear response. A, association phase of competition experiments with amoxicillin concentrations ranging from 0-2.4 μM. B, linear regression of % maximal response vs. amoxicillin concentration.

Environmental samples. Water samples collected from water and sewage treatment plants were analyzed with the assay. Comparative results are shown in Fig. 5A. As expected, stark differences in response were observed between raw and treated sewage and untreated lake water (the inlet for drinking water purification) and finished drinking water. Raw sewage gave a baseline response, indicating that the β-lactam concentration was sufficiently high to effectively bind all antibodies. Drinking water was almost indistinguishable from the buffer only control. It can be concluded that the plants are effectively reducing the concentration of amoxicillin and other β-lactams.
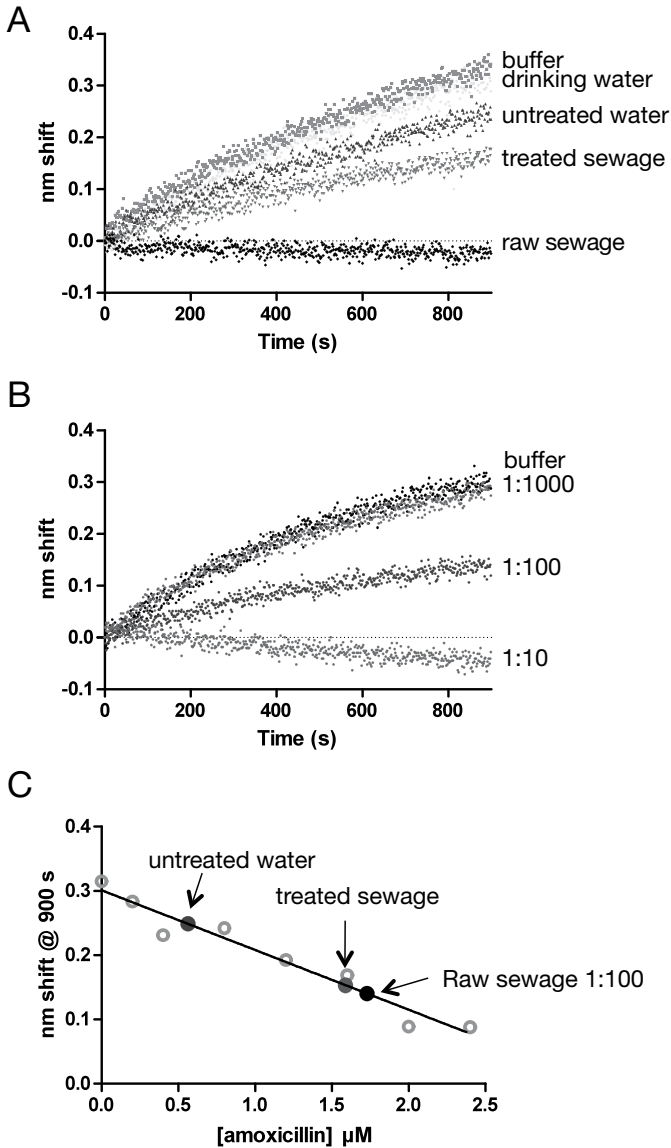
Figure 5. Analysis of environmental samples. A, Response to undiluted raw and treated sewage, untreated water (influent to water treatment plant) and drinking water. B, Tuning experiment to determine appropriate dilution of raw sewage. C, linear regression of response at 900s. Standards are shown as open circles. Positions of shifts at 900s for the experimental samples are shown as closed circles.

To determine the concentration of β-lactams in the sewage, a dilution series was performed to gauge an appropriate dilution to yield sensitivity within the established parameters (Fig. 5B). Tenfold dilution gave a baseline response whereas a 1:1000 dilution gave a near-maximal one. Dilution of 1:100 yielded a response between maximum and baseline, which at the end of the association phase reached 0.1426 nm shift. Plotting response at the end of association phase versus the responses of known amoxicillin concentrations yielded a linear relationship ($R^2 = 0.95$, slope = -0.093), allowing measurement without ambiguities inherent in determining plateaus of association curves (Fig. 4B). Determination is also simpler in that linear regression does not require either expensive software or numerous iterations. The choice of response at 900 s was somewhat arbitrary. Determination from response at shorter times was possible, *e.g.* linear regression of response at 600 s yielded an equally high correlation of $R^2 = 0.96$ with a slightly lower slope, -0.076 (data not shown). The measured concentration of amoxicillin in sewage was 170 µM when accounting for dilution (Fig. 5C). The responses of the other environmental samples undiluted yielded concentrations of 1.6 µM for treated sewage and 0.6 µM for lake water.

Regeneration. Sensors could be regenerated for additional use by brief, thrice-repeated exposure to glycine pH 2.0, but not without loss of response (Fig. 6). The loss of response is significant, about 33%, making regenerated sensors suitable only for qualitative measurements.
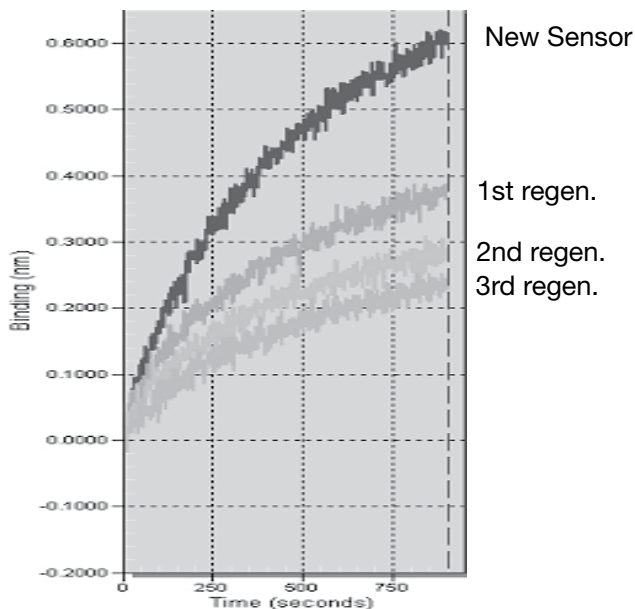
Figure 6. Regeneration of sensor recovers some activity. Regeneration protocol was executed followed by additional association experiments against the same analyte solution through 3 regenerations.

DISCUSSION

Advantages of Assay. The principal advantage of immunoassays over direct spectroscopic measurement is sensitivity. However, ELISA has the serious disadvantage of speed, often taking a day or more to collect data. Optical biosensor assays, like that of Cacciatore, *et. al.* (9) and ours, have the advantages of the high sensitivity of ELISA but are significantly faster. Hands-on time is in our BLI assay is minimal, about 15 minutes to set up each run. Crosslinking, baseline and data collection take less than an hour start-to-finish and are automated, as compared to hours (often with overnight incubation) and many manual steps for ELISA. The BLI assay has ease-of-use advantages over the SPR method of Cacciatore by avoiding the need for a carrier protein and the ability to simultaneously measure multiple samples; the BLI instrument can assay eight samples at once whereas SPR sensors require multiple analyte injections with surface regeneration steps between injections. HPLC also requires multiple injections. Moreover, SPR chip cost ~$150 and up, whereas BLI sensors are less than $5 each.

Further development. One shortcoming of the assay, as with any immunoassay, is cross-reactivity. Since the antibody used is reactive to cloxacillin and other β-lactams, the measured concentrations of the environmental samples are indicative of the presence not of amoxicillin exclusively, but of one or more β-lactam drugs. At present the assay is useful for qualitative determination of the presence of lactams and quantitation of samples known to contain only amoxicillin. Further development to characterize the degree of cross-reactivity of the antibody to other lactams is necessary to achieve true quantitative utility for complex samples.

While the crosslinking necessary to immobilize the BSA-AMOX requires mixing reagents moments before immobilization, scale-up of the assay for multiple samples is readily achievable. Sensor activation, loading and quenching can be done in batch with up to 96 sensors at a time (10). The instrument is capable of unattended operation; it can perform 8 simultaneous measurements, dislodge used sensors, pick another 8 and perform a different set of measurements and so on for up to 12 sets of 8 sensors. Since only association measurements need be made, up to 88 samples could be analyzed on the instrument in a single programmed series of runs. A setup for that assay would use precharged sensors with a sample plate containing 1 column of 8 wells for baseline determination and 11 rows of 8 samples each.

The assay should be readily adaptable to any small molecule for which antibodies or other binding partners are available and for which immobilization can be achieved. Moreover, non-aquatic samples can be used assuming extraction of the target molecule can be achieved in a buffer suitable for antibody binding, *e.g.* antibiotic-containing tissue samples could be homogenized in detergent containing buffer and fractionated so that the soluble fraction could be assayed. The assay should also be easily convertible to other biosensor platforms.

ACKNOWLEDGEMENTS

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