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Evanescent Wave Optical Fiber Sensors Using Enzymatic Hydrolysis on Nanostructured Polyaniline for Detection of β-Lactam Antibiotics in Food and Environment

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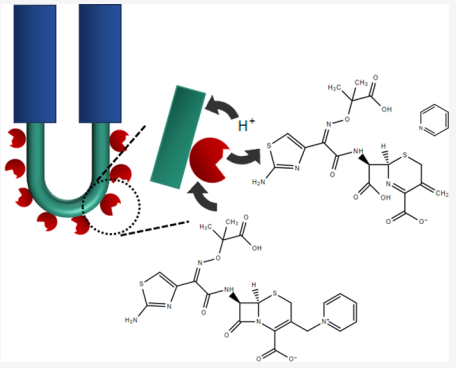
ABSTRACT: β-Lactam antibiotics such as penicillins and cephalosporins are extensively used for human infection therapy. Consistent unintended exposure to these antibiotics via food and water is known to promote antibiotic-resistant bacterial pathogenesis with high morbidity and mortality in humans. An optical enzymatic biosensor for rapid and point-of-use detection of these antibiotics in food and water has been developed and tested. Enzymatic hydrolysis of β-lactams, on the electroactive polyaniline nanofibers, altered the polymeric backbone of the nanofibers, from emeraldine base form to emeraldine salt, which was measured as an increase in evanescent wave absorbance at 435 nm. The sensors were calibrated by spiking antibiotic-free milk with ceftazidime (as a model β-lactam analyte) in a linear range of 0.36−3600 nM (R^2 = 0.98). The calibration was further validated for packaged milk, local cow milk, and buffalo milk. A similar calibration was devised for chicken meat samples in a linear range of 9−1800 nM (R^2 = 0.982) and tap water in a linear range of 0.18−180 nM (R^2 = 0.99). Interestingly, it was possible to use the same calibration for the determination of other β-lactam antibiotics (ampicillin, amoxicillin, and cefotaxime), which reflects the usefulness of the sensor for wide-scale deployment. The sensor performance was validated with a wastewater sample, from a wastewater treatment plant (WWTP), qualitatively analyzed by high-resolution liquid chromatography coupled with mass spectroscopy for detection of β-lactams. The sensor scheme developed and tested is of grassroot relevance as a quick solution for measurement of β-lactam residues in food and environment.



INTRODUCTION

The use of antibiotics as preventive medicine, to enhance productivity, quality, and durability of livestock produce, is a significant factor contributing to antimicrobial resistance (AMR).[1,2] As per the U.S. Food and Drug Administration[3] (FDA 2014), the annual estimated use of antimicrobials for livestock was 14.6 million kg, about 4 times the antibiotic use for human bacterial infection therapy (3.29 million kg).[4] As the use of antibiotics continues to increase worldwide,[5] most regulatory bodies are calling for gradual and voluntary phasing out of these compounds[6] in sectors where their use is not absolutely essential. In the face of increasing number of human casualties due to antimicrobial resistance,[7,8] it is recommended that the use of frontline antibiotics remains exclusive to healthcare. There do exist several regulations proscribing extralabel use of cephalosporins (except ceftiofur and cephapirin) in swine, chicken, turkey, and cattle.[9] However, in the absence of reliable point-of-care technologies for antibiotic monitoring, such regulations are frequently violated.

Hospital wastes, pharmaceutical wastes from industries, and human and animal excreta are major sources of antibiotic contamination, which promotes antimicrobial resistance in aquatic environments. Several studies report the occurrence and prevalence of β-lactam antibiotics in hospital wastewater, influents of wastewater treatment plants (WWTPs), and sewage-contaminated surface waters. The occurrence of β-lactam antibiotics in the range of 20−80 μg/L in hospital effluents has been reported by Kümmerer et al.[10] In another study by Mutiyar et al.,[11] the concentration of ampicillin in the influent of a sewage treatment plant in Delhi, India, was found to be in the range of 23.5−263.3 μg/L.

Growing consciousness and awareness toward food safety[12,13] have propelled the demand for antibiotic-free meat and milk, worldwide.[14,15] Conventional antibiotic testing in water, wastewater, and food generally involves a sample preconcentration step using solid-phase extraction followed by chromatographic separation coupled with mass spectroscopic analysis of antibiotics. The process comes at a premium of sophisticated analytical instrumentation and operator expertise, often unaffordable for small- or medium-scale industries, especially those in developing countries. Microbial inhibition-

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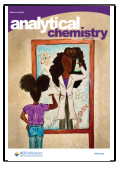

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based screening assays are one of the earliest and frequently used methods for the detection of antibiotic residues in food. There are several immunoassay and microbial inhibition assay-based commercial kits such as AuroFlow by PerkinElmer, Charm II by Charm Sciences, PremiTest by Ridacom, and Delvotest by DSM for the detection of antibiotic residues in food. However, most of these are incapable of precise quantification and do not offer adequate detection limits as discussed by Pikkemaat.[16]

Technologies in research for quantification of antibiotics in food and water samples use either aptamers,[17,18] antibodies,[19,20] enzymes,[21,22] whole cells (bacteria),[23,24] or molecularly imprinted polymers[25] as the biorecognition element on customized nanostructured substrates, such as quantum dots,[26] metal−organic frameworks,[27] plasmonic nanoparticles,[28] hydrogels,[29] and metamaterials.[30] The combination is then used on electrochemical,[31] optical,[32] mass-sensitive,[33] or hybrid[17] transduction platforms for the detection and quantification of antibiotics. Though promising, most technologies have scalability issues on diverse parameters such as cross-sensitivity, robustness, ease of operation, range, and detection limits. Various novel sensing and biosensing technologies reported in the literature for antibiotic detection are seldom tested for performance in complex organic matrices such as wastewater, milk, and food, limiting their point of use. Thus, despite limitations, standardized chromatography coupled with mass spectroscopy-based methods remain universally accepted gold standard techniques for detection and quantification of antibiotics. There is an urgent need to develop simple, robust, and point-of-use sensing systems for quantification of antibiotic residues in water and animal products that are directly consumed by humans, especially for those antibiotics used as frontline medicines for human infection therapy.

A well-known mechanism of antimicrobial resistance in bacteria, particularly to β-lactam antibiotics, is by secretion of β-lactam hydrolyzing enzymes called β-lactamases.[34] These enzymes hydrolyze the β-lactam ring of a β-lactam antibiotic, rendering the antibiotic incapable of binding on to the penicillin-binding proteins of the bacterial cell wall, and hence ineffective. Bacteria exposed to newer, potent classes of β-lactams, such as third-, fourth-, and fifth-generation cephalosporins and carbapenems, have evolved by upgrading to production of newer forms of β-lactamases (such as cephalosporinases and carbapenemases).[35] These diverse arrays of enzymes have been classified structurally into classes A−D in the Ambler's classification scheme[36] or functionally into groups 1−3 in the scheme proposed by Bush, Jacoby, and Medeiros.[37] There exist 4453 identified variants of β-lactamases, as reported in the β-lactamase database (September 2020).[38] This gives an extensive library of enzymes, which may potentially be utilized as bio-receptors for the development of enzymatic biosensors targeting specific β-lactam antibiotics.

Enzymatic hydrolysis of a molecule of β-lactam antibiotic is associated with the release of a proton, and/or byproducts that may be acidic in nature, (such as penicillin to penicillinoic acid or ampicillin to ampicilloic acid) causing a change in pH. However, the phenomenon is highly localized at the site of antibiotic degradation, especially with trace levels of antibiotics. In the present work, we devised a polyaniline nanofibers coated U-bend optical fiber-based sensing strategy for measuring this change in pH, and correlating the same with a cumulative concentration of β-lactam antibiotics present in a sample. Extended-spectrum transmission electron microscopy (TEM) β-lactamase (named after Temoniera, the patient first found to carry β-lactamase producing resistant strain of *Escherichia coli*[39]) with activity toward common β-lactam antibiotics: penicillins, cephalosporins, and monobactams (as specified by manufacturer datasheet), used for human infection therapy, was used as the biorecognition element in this study. The enzyme was immobilized on an electroactive, pH-sensitive polymer, polyaniline. Enzymatic hydrolysis of the β-lactam antibiotic protonated the polyaniline nanofibers altering its charge distribution and the oxidation state from emeraldine base (EB) to emeraldine salt (ES) form. This caused evanescent wave modulation in the polyaniline nanofibers modified U-bend region of the optic fiber and concentration-dependent absorbance change at 435 nm. The sensor was tested for detection of antibiotic residues in cow milk, buffalo milk, packaged milk, chicken meat, and tap water. The sensor was further tested to evaluate the total β-lactam load in raw wastewater samples, qualitatively screened for β-lactam antibiotics through high-resolution liquid chromatography coupled with mass spectroscopy (HRLC-MS/MS). The sensor response was robust across various milk and water matrices with a limit of detection of 0.18 nM. Although the concept of enzymatic protonation and deprotonation for biosensing applications is not new,[40] this, to the best of our knowledge is the first report on the development and testing of an evanescent wave-based enzymatic optical biosensor for detection of antibiotics across real food, water, and wastewater samples in a range relevant for point-of-use sensing.

EXPERIMENTAL SECTION

Reagents. Ammonium persulfate (APS), ampicillin, aniline, glutaraldehyde, levofloxacin, and doxycycline were procured from Sigma-Aldrich, Merck, India. Ceftazidime pentahydrate and erythromycin were purchased from HiMedia Laboratories Private Limited, India. Cefotaxime sodium salt and amoxicillin trihydrate were procured from Sisco Research Laboratories, India. Bovine serum albumin (BSA) was purchased from GeNei, Merck, India. Recombinant *E. coli* β-lactamase TEM precursor protein, with established activity against penicillins and cephalosporins was procured from Abcam, U.K. All aqueous solutions were prepared using deionized water from a Milli-Q filtration plant (18.2 MΩ cm).

Optical Fiber Preparation. High −OH, silica core, and multimode optical fibers of core diameter 200 μm were procured from Thorlabs. Optical fibers were cut into 40 cm long strips, and the ends of individual fibers were polished with a 5 μm grit, silicon carbide lapping sheet, Thorlabs. A 2 cm portion of the central region of the fiber was dejacketed, decladded, and bent to a "U" shape using a butane flame. The bend diameter was maintained at 1.5 mm to ensure optimum sensitivity.[41] The U-bent fibers were cleaned by successive incubation in 1 M sodium hydroxide for 30 min, 1:1 (v/v) mixture of methanol and hydrochloric acid for 60 min, and sulfochromic acid for 10 min. The optical detection assembly is discussed in the Supporting Information (Section S1).

For modification of the optical fiber with polyaniline nanofibers,[42] the cleaned U-bend region was initially incubated in 50 mM of the monomer, aniline (reconstituted in 1 M HCl). To this, an equal volume of 50 mM APS (also reconstituted in 1 M HCl) was added as the redox polymerization initiator. The polymerization mixture gradually

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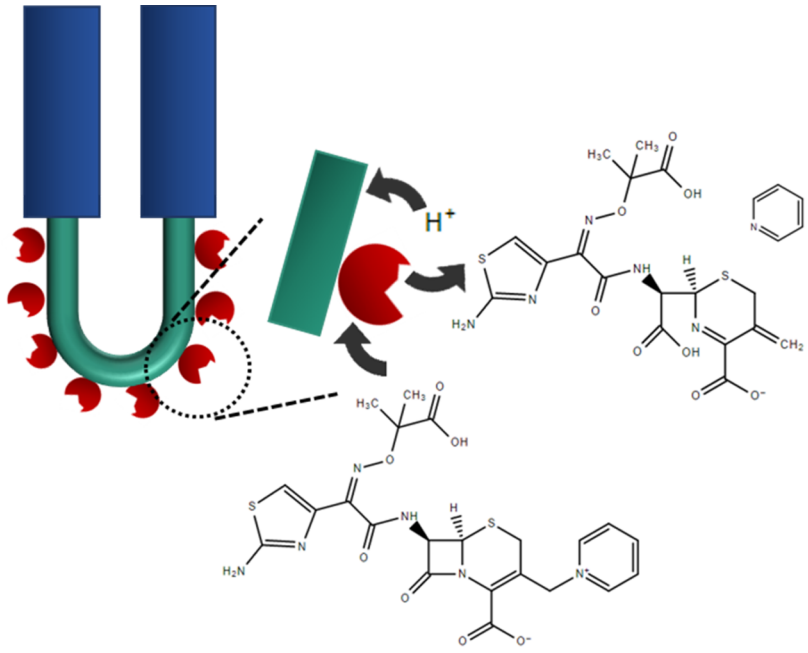

Figure 1. Schematic representation of the mechanism of sensing of β-lactam antibiotics with polyaniline-modified U-bend optical fiber sensors (example shown with ceftazidime, which, is hydrolyzed to release a proton).

changed color from colorless to light pink to blue to green, confirming the oxidative polymerization of aniline. Immobilization of the same on the optical fiber was monitored online till an optical density (OD) of 2 (at 650 nm) followed by immediate washing of the optical fiber with 1 M HCl to wash away unreacted monomer and loosely adhered polyaniline nanofibers. The absorbance spectrum and time-resolved spectral response for polyaniline deposition on optical fibers are illustrated in Section S2 of the Supporting Information. The optical density of polyaniline nanofibers deposited on optical fibers plays an important role in the sensitivity of the sensor. Deposition of polyaniline nanofibers was experimentally optimized to an OD of 2 to attain an optimal signal sensitivity with a minimal evanescent wave absorbance noise. This is discussed further in Section S7.

β-Lactamase enzyme was immobilized on polyaniline-coated optical fiber by covalent cross-linking with glutaraldehyde (Figure 1). Polyaniline-coated fibers were incubated in 1% (v/v) aqueous glutaraldehyde solution for 40 min, followed by incubation in 120 IU/mL of TEM β-lactamase (reconstituted in deionized water as per manufacturer's datasheet) for immobilization of the enzyme. The remaining amine capture sites on the fiber were blocked with 2 mg/mL bovine serum albumin, reconstituted in 10 mM phosphate buffer saline, pH 7 (Section S4). Binding of enzyme and BSA were monitored online and recorded as an increase in absorbance with a peak at 600 nm (Section S5). All measurements were performed in triplicate on three different optical fiber probes, modified as per the stated scheme. Error bars are indicative of the standard error of a particular measurement set.

Analysis of Antibiotics in Milk and Wastewater. Packaged milk, cow milk, and buffalo milk were procured from a local store and analyzed for antibiotic content using HRLC-MS/MS (Agilent Technologies) in auto-MS/MS positive electrospray ionization (ESI) mode. For analysis, the milk samples were preprocessed as described in Du et al.[43] to remove fat and run through a C-18 chromatographic column (Hypersil GOLD, 100 × 2.1 mm, 3 μm) at a flow rate of 0.3 mL/min. Correlation of the peak m/z values with the pharmaceutical database Metlin-B.06, Agilent Technologies, showed that no β-lactam antibiotics were present in the milk samples tested.

The composite raw wastewater sample (24 h composite sample) was collected in amber-colored glass bottles directly from a WWTP located in the western part of India. U.S. Environmental Protection Agency (U.S. EPA) protocol was followed for sample collection, transportation, and storage.[44] Soon after sample collection, 500 mL of raw wastewater sample was filtered, followed by solid-phase extraction (SPE) as per Padhye et al.[45] The preconcentrated sample was analyzed through HRLC-MS/MS (Agilent Technologies) in auto-MS/MS positive ESI mode coupled with a reversed-phase Phenomenex MAX-RP column (Phenomenex MAX-RP (250 mm × 4.6 mm, 5 μm)). The gradient elution in chromatographic analysis was performed using a mobile phase comprising water (supplemented with 0.1% formic acid (FA) and acetonitrile (ACN)) at a flow rate of 0.3 mL/min for 60 min. The mobile phase program included five stages with a variable ramp rate of ACN: increase in ACN from 10 to 30% over 10 min, held constant at 30% up to 30 min, increase in ACN up to 90% till 50 min, reduction of ACN to 10% at 52 min, and held constant at this level up to 60 min.[46] Finally, the peaks were analyzed with the pharmaceuticals database (Metlin-B.06, Agilent Technologies) to obtain qualitative information about the presence of β-lactam antibiotics (Section S9). Both MS/MS fragmentation pattern and mass tolerance of ±5 ppm were used as the qualifying criteria for the identification of such antibiotics.

RESULTS AND DISCUSSION

Characterization. Polyaniline nanofibers synthesized by oxidative polymerization of aniline were analyzed by a transmission electron microscope (TEM, JEOL-JEM-2100F) for morphology. The synthesis of amorphous polyaniline

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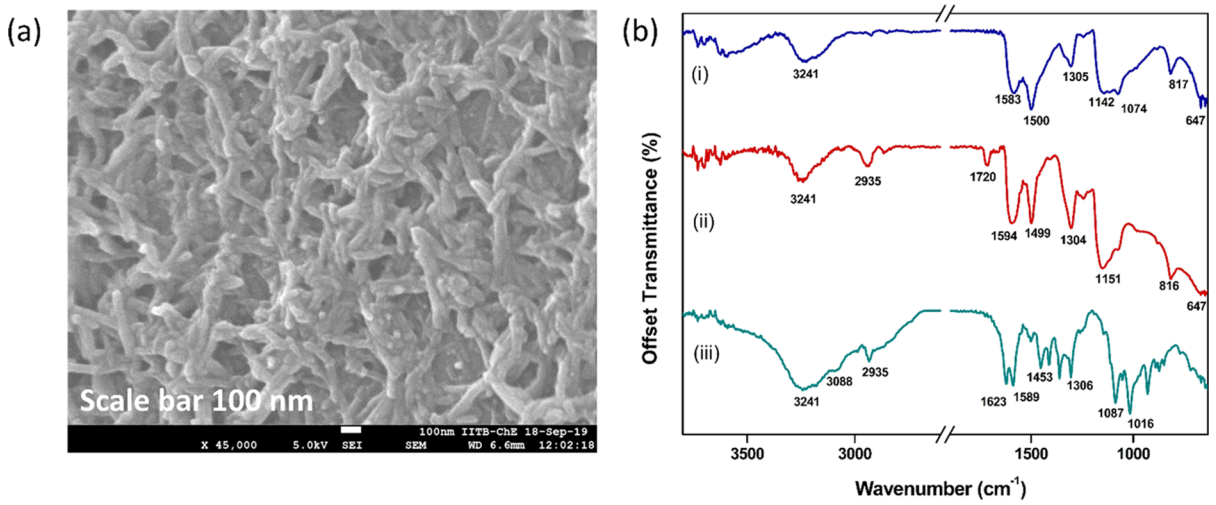
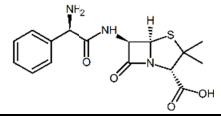
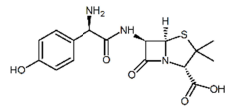
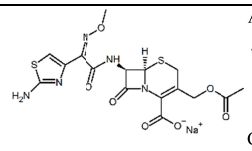
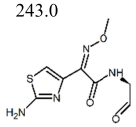
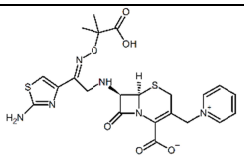
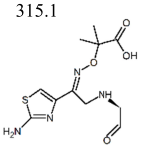


Figure 2. (a) FEG-SEM analysis of polyaniline nanofiber-coated optical fiber. (b) Fourier transform infrared (FTIR) spectrum of (i) polyaniline, (ii) glutaraldehyde-modified polyaniline, and (iii) enzyme-immobilized, glutaraldehyde-modified polyaniline.

Table 1. Peak Analysis of the Mass Spectrum of Certain β-Lactam Antibiotics Dissolved in Deionized Water and That Incubated in Enzyme TEM β-Lactamase[a,b]

Antibiotic (Molecular weight) (g/mol)	Structure	Fragment X	$[M+H]^+$	$[M+Na]^+$	$[M-X+H]^+$	$[M-X+Na]^+$	$[M_h+H]^+$	$[M_h+Na]^+$	$[M_{h/d}+H]^+$	$[M_h-X+H]^+$	$[M_h-X+Na]^+$	$[M_{h/d}-X+H]^+$	Other abundant fragment ion
Ampicillin (349.4)		-	350.1	372.1	-	-	368.1	390.1	324.1	-	-	-	-
Amoxicillin (365.4)		NH_2	-	-	350.1	372.1	-	-	-	368.1	390.1	324.1	-
Cefotaxime Sodium (477.5)		Acetyl group C_2H_3O	456.0	478.0	396.0	-	-	-	-	414.0	-	370.0	243.0 
Ceftazidime (546.6)		Pyridine C_5H_5N	547.0	-	468.0	-	-	-	-	486.1	-	442.1	315.1 

[a] $[M]^+$: molecular ion (m/z), $[M + H]^+$: hydrogen adduct (m/z), $[M + Na]^+$: sodium adduct, $[M_h]^+$: hydrolyzed molecular ion (m/z), $[M_{h/d}]^+$: hydrolyzed decarboxylated molecular ion (m/z). [b] Columns with headings highlighted in gray represent hydrolyzed antibiotics product ions (in m/z).

nanofibers of approximate width 40 nm was confirmed as illustrated in Section S3. Polyaniline nanofiber-coated optical fibers were analyzed for surface coverage and morphology with field emission gun scanning electron microscopy (FEG-SEM, JEOL JSM 7600F), as shown in Figure 2a.

Fourier transform infrared (FTIR) spectroscopy of polyaniline nanofibers washed with deionized water, after glutaraldehyde treatment and post enzyme immobilization on polyaniline was performed with a 3000 Hyperion microscope with a Vertex 80 FTIR system (Buker, Germany) to confirm modification of polyaniline with glutaraldehyde and subsequent immobilization of enzyme (Figure 2b(i–iii), respectively). Peaks at 1500 and 1583 cm^{-1} correspond to the C=C stretching vibrations of benzenoid and quinoid rings of polyaniline.[47] The sharp peaks at 1305 and 1142 cm^{-1} can be attributed to the C–N stretch of secondary aromatic amine of polyaniline. The peak at 1074 cm^{-1} can be ascribed to the C–C stretching of the alkyl chain of the polymer, while a peak

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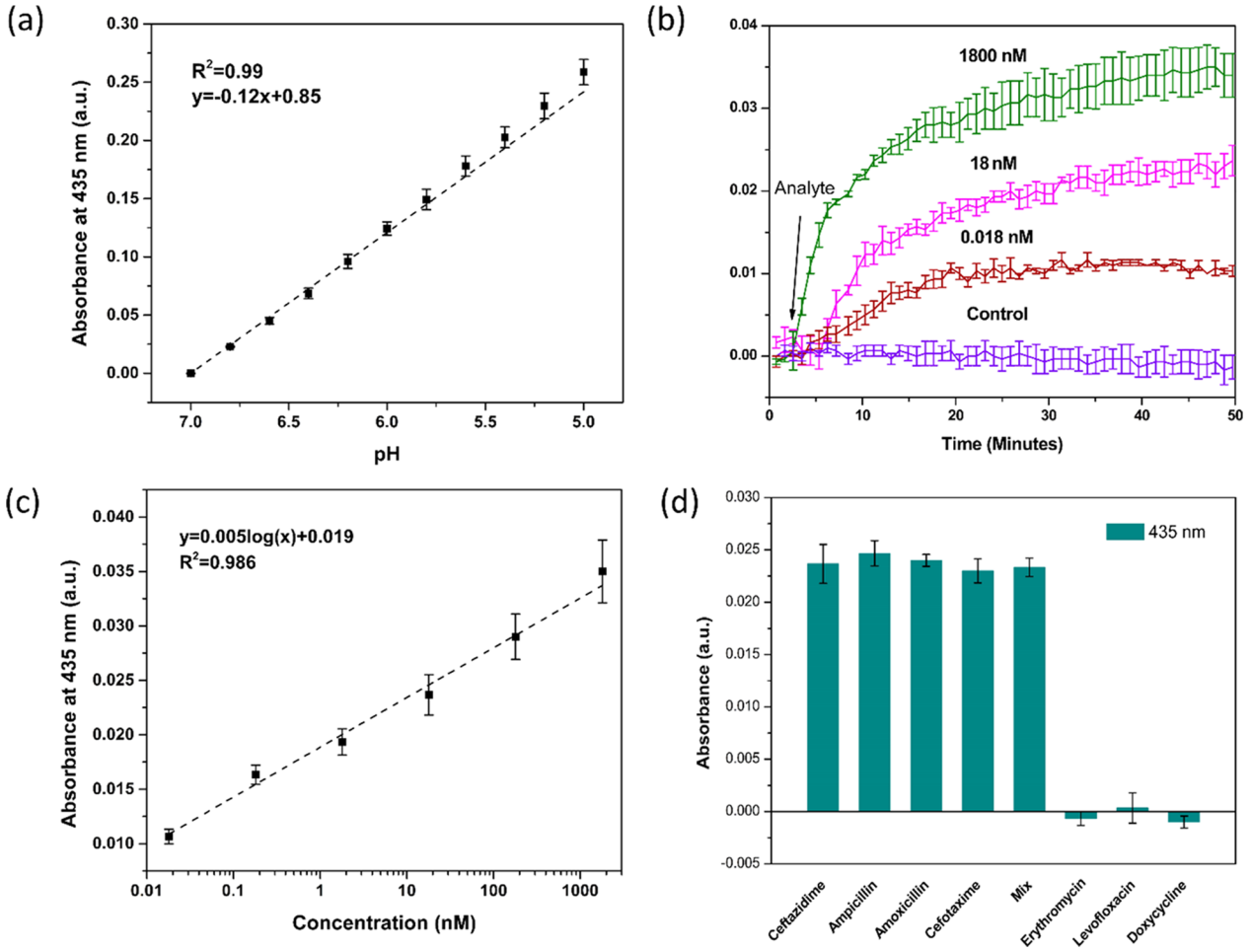

Figure 3. (a) Change in absorbance of polyaniline-coated optical fibers for varying pH; (b) time-varying response of the sensor to different concentrations of ceftazidime (prepared in deionized water), control represents sensor response to deionized water; (c) calibration curve for ceftazidime in distilled water; and (d) response of the sensor toward certain β-lactam, non-β-lactam, and mixture of antibiotics. The pure antibiotics were used at an 18 nM concentration. "Mix" is a solution consisting of β-lactam antibiotics (6 nM of ceftazidime, ceftriaxone, and ampicillin individually—a cumulative β-lactam concentration of 18 nmol), non-β-lactam antibiotics (18 nM of levofloxacin, doxycycline, and azithromycin individually), and 18 nM of random constituents such as an analgesic (paracetamol) and urea. Absorbance at 435 nm shows that the sensitivity of the sensor is toward β-lactam antibiotics only.

at 1244 cm^{-1} is correlated to the C−C twisting of the same.[48] Vibration associated with the bending of C−H on the 1−4 disubstituted ring appears as a peak at 817 cm.[49] All of the peaks obtained are in agreement with that previously reported. Modification of polyaniline with glutaraldehyde did not cause much alteration in most of the peaks. However, additional sharp peaks at 1720 and 2935 cm^{-1} were observed, which could be attributed to −C═O stretching of the free aldehyde and −CH stretching, respectively, thus confirming glutaraldehyde modification.[50] Finally, binding of enzyme to glutaraldehyde-modified polyaniline resulted in several additional peaks and masking of certain peaks. A peak at 1622 cm^{-1} can be attributed to the C═O vibrations of amide I linkage of the enzyme. A broad peak from 3100 to 3400 cm^{-1} is attributed to the characteristic amide A and amide B band of the enzyme. The absence of the peak at around 1140−1150 cm^{-1} may be due to the masking of the secondary aromatic amine signature of polyaniline by the enzyme.[51]

The catalytic hydrolysis byproducts of some of the commonly used β-lactam antibiotics (ampicillin, amoxicillin, ceftazidime, and ceftriaxone) by the chosen enzyme were studied by quadrupole time-of-flight mass spectroscopy (Q-TOF MS), Agilent Technologies, in positive ESI mode. This was done to confirm the activity of the enzyme against penicillins and cephalosporins and the formation of acidic byproducts. The antibiotics were dissolved in deionized water to a concentration of 1 mg/mL and incubated with β-lactamase for 2 h. An analysis of the peaks of the mass spectrum of enzyme-treated antibiotics confirmed hydrolysis and is briefly summarized in Table 1. A more detailed analysis of the mass spectrum is presented in the Supporting Information (Section S6).

Enzymatic Hydrolysis and Selectivity Studies. A unit of the emeraldine base form of polyaniline comprises two quinoid imines and two benzenoid amines. The two quinoid imines, being susceptible to protonation, along with the two benzenoid amines, undergo rapid protonation in acidic solutions to form emeraldine salt. This transformation of polyaniline from EB to ES can be seen as a change in its UV−visible spectra varying with different degrees of protonation in solution with varying pH. The sensitivity of polyaniline nanofiber-modified optical fibers in the pH range of 7−4 was studied as a change in the absorbance spectrum from 430 to 750 nm. The optical fibers were referenced at pH 7 and

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Table 2. Recovery Studies of the Sensor[a]

sample details	concentration of β-lactam antibiotic spiked (nM)	concentration of antibiotic found	recovery (%)	coefficient of variation (n = 3)
CAZ + CTX + AMP in deionized water	0.018	0.021	116.7	10.8
CAZ + CTX + AMP + three other non-β-lactam antibiotics + paracetamol (analgesic) + urea in deionized water	1.8	1.6	88.9	8.1
	0.018	0.016	88.9	10
CAZ in cow milk	18	19.6	108.9	13.6
	1.8	1.8	100	11.1
CAZ in buffalo milk	18	16	88.9	14.2
	1.8	1.5	83.3	14.7
CAZ in packaged milk	1.8	1.5	83.3	6.2
AMP in organic milk	28	30.6	109.3	13.7
	2.8	2.6	92.9	12.5
CAZ in chicken meat	90	82.4	91.6	8.8
AMX in chicken meat	54	55.82	103.3	8.53

[a]CAZ: ceftazidime, CTX: ceftriaxone, AMP: ampicillin, AMX: amoxicillin.

exposed to solutions with progressively lower pH solutions. The decrease in pH resulted in an increase in absorbance in the range 430−460 nm and a decrease in absorbance at other wavelengths (Section S8). Bulk pH sensitivity of the sensor was thus calculated in terms of increase in absorbance at 435 nm, per unit decrease in pH and was found to be 0.012 au per 0.1 unit decrease in pH (Figure 3a).

This pH sensitivity of polyaniline was utilized to develop an enzymatic optical sensor for detection of β-lactam antibiotics with TEM β-lactamase as the biorecognition element. TEM β-lactamases are serine hydrolases. The hydroxyl group of serine amino acid attacks the carbonyl carbon of the amide bond in the β-lactam ring of an antibiotic and forms an acyl-enzyme intermediate. This is followed by subsequent hydrolysis of the intermediate to form various degradation products and a proton. Generation of a proton and acidic products due to hydrolysis results in a change in localized pH in the vicinity of polyaniline and thereby an increase in absorbance at 435 nm. This primarily corresponds to the pH-sensitive cation radical of the ES form of polyaniline. Figure 1 shows a schematic representation of the sensing mechanism of ceftazidime, a third-generation cephalosporin. Ceftazidme is hydrolyzed by the immobilized enzyme to form an exomethylene intermediate, pyridine, and a proton that causes protonation of polyaniline and a change in absorbance.

The response of TEM β-lactamase-immobilized optical fibers to different concentrations of β-lactams dissolved in deionized water was studied. The response of the sensor toward different concentrations of ceftazidime (a model β-lactam antibiotic) in the range 0.018−1800 nM was tested. Enzyme-immobilized optical fibers were incubated in ceftazidime solutions, and the increase in absorbance at 435 nm was recorded for 50 min. A linear calibration curve with R^2 = 0.988 and an experimental detection limit of 0.018 nM was obtained as depicted in Figure 3b. The time-resolved spectral response of the sensor to different concentrations of ceftazidime is illustrated in Figure 3c. The response of the sensor toward 18 nM of some commonly used β-lactam antibiotics like ampicillin, amoxicillin, and ceftriaxone was also evaluated and is shown in Figure 3d. The results indicate that the sensor calibration for ceftazidime holds for all of the aforesaid β-lactam antibiotics.

Sensor selectivity studies were performed with a few non-β-lactam antibiotics such as levofloxacin (a fluoroquinolone), erythromycin (a macrolide), and doxycycline (a tetracycline). A negligible increase in absorbance at 435 nm was seen for these, and hence, it was ascertained that the response of the sensor was exclusive to enzymatic hydrolysis of specified β-lactam antibiotics (Figure 3d). A real wastewater matrix is likely to contain a mixture of β-lactam, non-β-lactam antibiotics, miscellaneous pharmaceuticals, and several other organic and inorganic components. To evaluate the sensor performance for such complex samples, the sensor was subject to a mixture of β-lactam antibiotics, non-β-lactam antibiotics, and random constituents such as an analgesic (paracetamol) and urea (Figure 3d). The change in absorbance was found to be comparable to the cumulative response as seen in the case of individual β-lactam antibiotics. Recovery studies were carried out in similar mixtures with cumulative β-lactam concentrations of 0.018 and 1.8 nmol. A recovery of 89% with respect to initial calibration was obtained for both samples (Table 2). The sensor was also subjected to a solution consisting of 0.006 nM ceftazidime, ceftriaxone, and ampicillin individually (a cumulative concentration of 0.018 nM of β-lactam antibiotics in deionized water), and the response was recorded. A recovery of 116.7% with a coefficient of variation (CV) of 10.8 was obtained (Table 2). Sensor recovery studies confirm the use of the same calibration for the estimation of antibiotics in contaminated water matrices of diverse organics.

Detection of Antibiotic Residues in Milk. The sensor was tested for detection of β-lactam antibiotic residues in locally procured cow milk, buffalo milk, and packaged milk. For sensor calibration, antibiotic-free organic milk was procured from Godrej Tyson Foods Ltd., Mumbai, India. Antibiotics were spiked in whole milk, and detection was carried out. Although the characteristic signature of enzymatic hydrolysis was recorded at 435 nm, repeatability studies showed large standard deviations across different sources of milk and low sensitivity. Therefore, it was necessary to incorporate a minimal preprocessing of milk, involving removal of fat, prior to sensing. The milk samples were processed so as to remove the fat, according to a method previously reported by Du et al.[43] Briefly, milk samples were first spiked with different concentrations of ceftazidime followed by removal of fat with 1% (w/v) trichloroacetic acid. The acid-treated milk was centrifuged, and the supernatant was used for further experiments.

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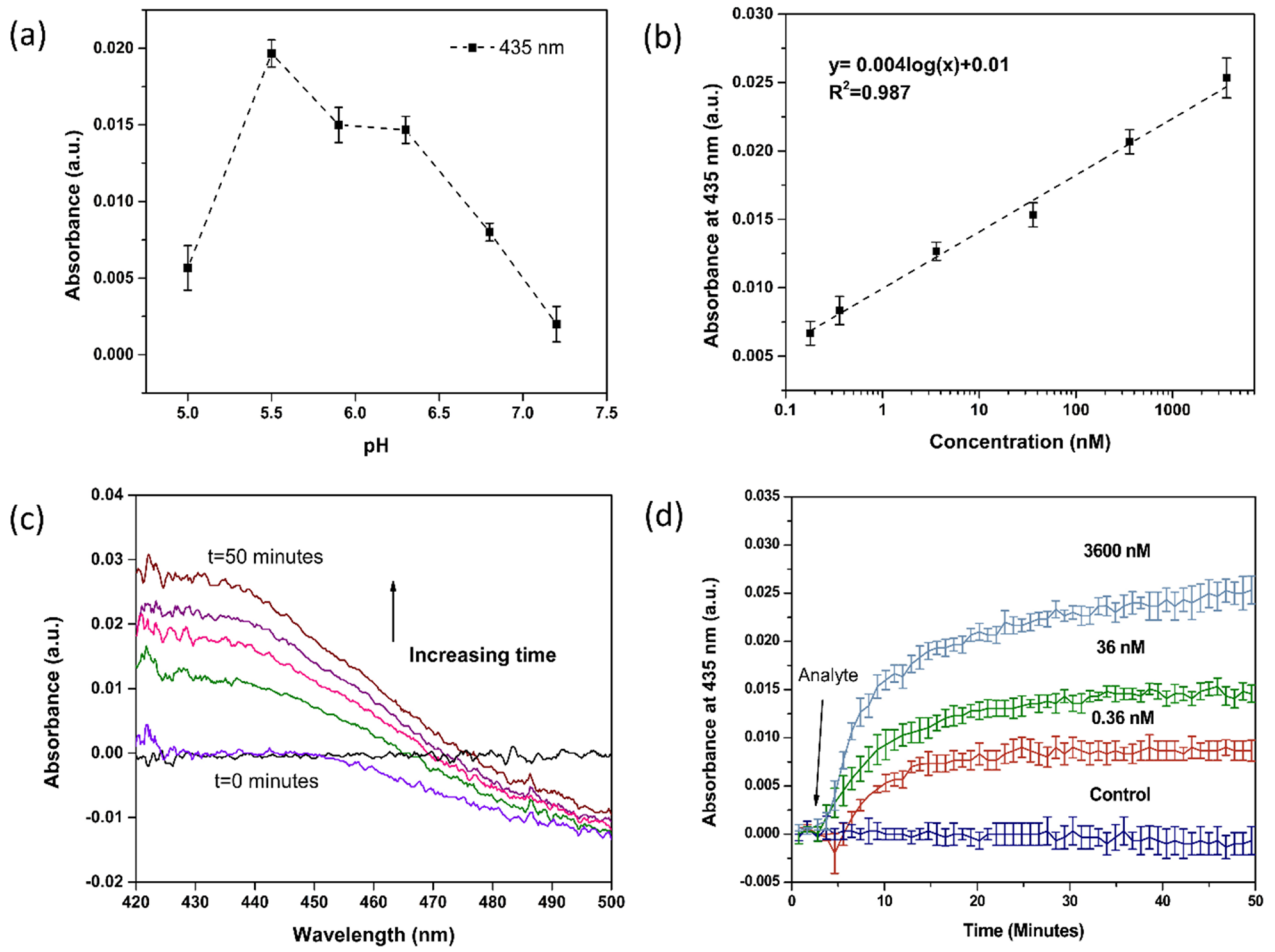

Figure 4. (a) Response of the sensor to 360 nM ceftazidime (in organic milk) at various pHs. (b) Time-varying response of the sensor to different concentrations of ceftazidime in milk. (c) Change in absorbance spectrum of the sensor in the presence of 1800 nM ceftazidime in milk. (d) Calibration curve of ceftazidime in organic milk.

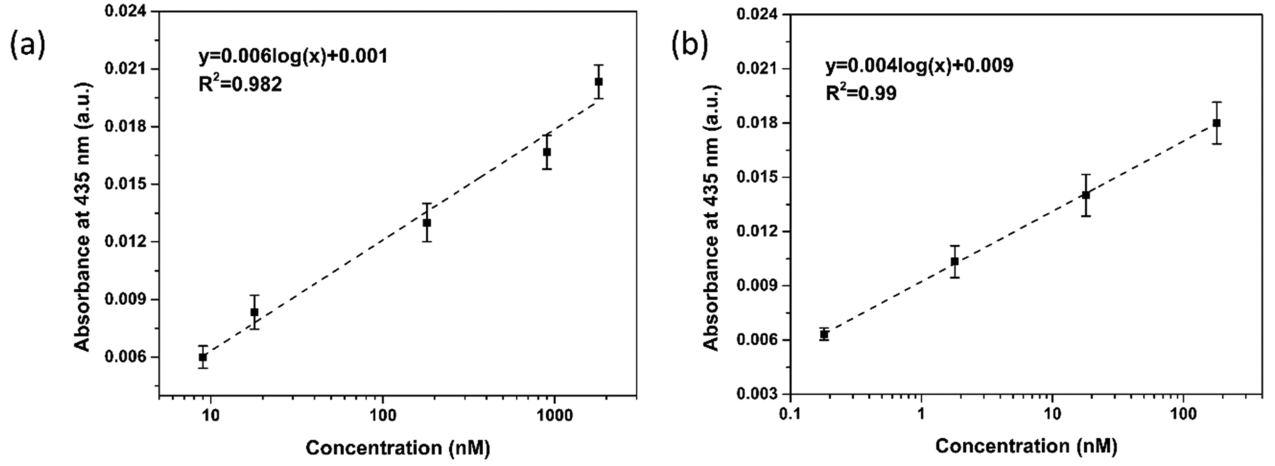

Figure 5. (a) Calibration curve obtained for different concentrations of ceftazidime spiked in antibiotic-free chicken. (b) Calibration curve obtained for different concentrations of ceftazidime spiked in tap water.

The activity of β-lactamase post immobilization on polyaniline nanofibers was evaluated at different working pHs. The pH of the milk samples spiked with 360 nM ceftazidime was adjusted to 5, 5.5, 5.9, 6.3, 6.7, and 7.2, and the response of the sensor was recorded for 50 min. The maximum change in absorbance at 435 nm was observed at a working pH of 5.5, while responses at pH 5 or 7.2 are lesser as shown in Figure 4a. Hence, pH 5.5 was selected for the detection of antibiotic residues using the proposed enzymatic optical sensor across all food and water matrices.

A linear calibration curve in the range of 0.18−3600 nM, with R^2 = 0.98 and an experimental limit of detection of 0.18 nM (Figure 4b) was obtained for ceftazidime spiked in antibiotic-free organic milk (maximum residue limit (MRL):

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Table 3. Comparative Analysis of the Recent Developments for Detection of β-Lactam Antibiotics

method	receptor	β-lactam antibiotic detected	experimental LoD	range	real samples tested	ref
colorimetric	quercetagetin coated AgNPs	amoxicillin	4.46×10^{-6} M	10×10^{-6}–95×10^{-6} M	tap water, blood plasma	53
fluorescence	antibody	cefquinome	0.5×10^{-9} M	0.5×10^{-9}–6.8×10^{-9} M	milk	19
SPR	MIP	amoxicillin	theoretical LoD: 0.073×10^{-9} M	tested range: 0.1×10^{-9}–2.6×10^{-9} M	tap water	33
electrochemical	aptamer	ampicillin	1×10^{-6} M	5×10^{-6}–5000×10^{-6} M	serum, saliva, milk	54
electrochemical	penicillinase	penicillin	0.1×10^{-12} M	1.25×10^{-13}–7.5×10^{-3} M	none	56
electrochemical	β-lactamase	benzylpenicillin	0.79×10^{-7} M	0.26×10^{-6}–0.66×10^{-6} M	milk	55
electrochemical	aptamer	ampicillin	0.01×10^{-9} M	0.1×10^{-9}–1×10^{-3} M	lake water	18
FET	enzyme-penicillinase	penicillin	$<200 \times 10^{-6}$ M	200×10^{-6}–25×10^{-3} M	urine	22
this work	enzyme-β-lactamase	ampicillin, amoxicillin, ceftazidime, cefotaxime	0.18×10^{-9} M in milk and water, 9×10^{-9} M in chicken meat	0.18×10^{-9}–1800×10^{-9} M (milk and water) 9–1800 nM (chicken meat)	milk, chicken meat, tap water, wastewater from WWTP	

10 μg/L corresponding to 18 nM ceftazidime, Food Safety and Standards Authority of India (FSSAI)). The change in the spectral absorbance response of the sensor when subjected to 1800 nM ceftazidime in milk and the time-resolved response of the sensor are shown in Figure 4c,d, respectively. The response of the sensor to ceftazidime and other β-lactam antibiotics spiked in different types of milk was also tested to check the applicability of the obtained calibration curve across diverse milk matrices. The milk samples were analyzed with HRLC-MS/MS and were found not to contain any β-lactam antibiotics. Recovery studies with the sensor were hence carried out with 18 and 1.8 nM ceftazidime spiked in cow, buffalo, and packaged milk, respectively. A good recovery with a maximum error of 20% across diverse milk matrices was obtained. The response of the sensor to 2.8 and 28 nM ampicillin, spiked in organic milk, was also evaluated (Table 2).

Detection of Antibiotic Residues in Meat. Antibiotic feed-free minced chicken, purchased from Godrej Tyson Foods Ltd., Mumbai, India, was used to test the applicability of the sensor for detection of β-lactam antibiotics in meat. Ceftazidime, a cephalosporin banned for use in poultry by FDA, European Union, was used for sensor calibration. Meat samples were prepared as reported by Marchesini et al.[52] Briefly, 100 μL of ceftazidime solution of various concentrations was added to 2.5 g of chicken samples and mixed thoroughly to obtain spiked chicken samples with antibiotic concentrations of 9–1800 nM. The samples were homogenized with 25 mL of deionized water for 15 min. The homogenate was collected, centrifuged, filtered, and the pH was adjusted to 5.5 for further experiments. The response of the sensor to different concentrations in spiked chicken samples was recorded in triplicate, and a linear calibration curve with $R^2 = 0.982$ was obtained as shown in Figure 5a. The experimental limit of detection for ceftazidime was found to be 9 nM. Sensor recovery studies were performed by spiking 90 nM ceftazidime and 54 nM amoxicillin in antibiotic-free meat. A recovery of 91.6% with a coefficient of variation (CV) of 8.8 and 107.7% with a CV of 8.53 was obtained, respectively (Table 2). This indicated good fitness of the sensor for diverse usage.

Detection of Antibiotics in Water and Wastewater. The sensor was tested for its applicability for the detection of β-lactam antibiotic residues in water and wastewater matrices. Regular tap water (Mumbai Municipal Corporation) was collected from Indian Institute of Technology Bombay, and raw wastewater samples were obtained from a wastewater treatment plant in the western part of India. Tap water was filtered with a 0.2 μm syringe filter to remove large particulate and organic matter (if present) and pH-adjusted to 5.5 for maximal sensitivity. To this, ceftazidime was spiked so as to obtain final antibiotic concentrations in the range of 0.18–180 nM (at decade intervals). A linear calibration curve with $R^2 = 0.99$ and an experimental limit of detection of 0.18 nM were obtained as shown in Figure 5b.

The sensor response was validated with wastewater samples, qualitatively analyzed by HRLC-MS/MS. The list of identified β-lactams along with their mass error, matched score with the database, molecular formula, molecular ion (m/z), and retention time (RT) is shown in Table S1. The mass error and database-matched score for the identified antibiotics were in the range of ±5 ppm and 51–87, respectively. It would be interesting to conduct additional experiments for exact quantification of identified β-lactams through LC-MS/MS followed by their validation with this sensor. However, this would require extensive analysis involving isotopes of all of the individual antibiotics, which is beyond the scope of this study. The estimated concentration of cumulative β-lactam antibiotics in the wastewater through the developed sensor was found to be 32.4 nmol with a CV of 4.6%. The time-varying response of the sensor to the wastewater is plotted in Section S10. The obtained detection limit of 0.18 nM in water is much lower than the concentrations of β-lactam antibiotics previously reported to occur in various wastewater streams. A comparative analysis of our sensor with other previously reported sensors for the detection of β-lactam antibiotics is tabulated in Table 3.

Sensor Reusability Studies. Since the developed sensor works on the principle of catalytic hydrolysis of β-lactam antibiotics by β-lactamases, the reusability of the sensor for detection of antibiotic residues was evaluated. Fat-removed, antibiotic-free milk was spiked with 1.8 nM ceftazidime, and the response of the same sensor to multiple measurements of the same analyte was performed. The sensor was then deprotonated by subjecting the sensor to the reference solution, that is, unspiked milk. Subsequently, the sensor response was recorded three more times for the same concentration of antibiotic. The experiment was repeated in triplicate using three different sensors to get the average response. Error with reference to the first reading and the coefficient of variation was calculated (Section S11). With errors of 3.45 and 13.79% from the initial reading for the

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second and third reuses, it was inferred that one sensor could be used twice.

CONCLUSIONS

An evanescent wave-based optical fiber sensor capable of point-of-use quantification of penicillins and cephalosporins, antibiotics that are commonly used for human therapeutics, was developed and tested. The sensor was tested with diverse organic matrices of milk, chicken meat, water, and real wastewater samples. The sensor calibration for each matrix could be used to detect a cumulative load of penicillins and cephalosporins, irrespective of the point of collection or source. The experimentally achieved limit of detection was 0.18 nM in milk (well below the MRL of 10 μg/L, which corresponds to 18 nM ceftazidime, FSSAI), 9 nM in chicken (MRL: 10 μg/kg which corresponds to 18 nM ceftazidime, FSSAI), and 0.18 nM in water. The designed biosensor required a minimal sample preparation step for removal of fat and was demonstrated for the measurement of cephalosporins and penicillins in local cow milk, buffalo milk, and packaged milk, using a single calibration. The obtained calibration curve can be used by any user for direct estimation of the antibiotics in any milk sample. With a detection limit of 0.18 nM, the sensor can also detect β-lactam antibiotics in any wastewater sources and sewage-contaminated surface waters, where a much higher concentration of these antibiotics has been reported. The sensor response was also found to be 86% of the original response, for measurements conducted after a week of immobilization, when stored at 4 °C. Thus, this bio-inspired biosensing technique has great potential for monitoring antibiotic concentrations in food and water. Since the principle of enzymatic hydrolysis of β-lactams by β-lactamase involves a change in pH universally, a different set of β-lactams from that reported here may be easily targeted merely by using an appropriate β-lactamase enzyme. The sensor is of utility to livestock and poultry farms and food processing industries for quality testing. It would also be a useful tool for pollution control boards and regulatory bodies for regulation of antibiotic use. The sensor would also be of great utility to common effluent treatment plants and WWTPs, for monitoring the presence of β-lactam antibiotics in the influent and their removal in the treatment process.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c04169.

Optical detection assembly, detailed mass spectroscopy analysis of antibiotics hydrolysis, data associated with deposition of polyaniline nanofibers, immobilization of enzyme and BSA on optical fibers, detection of β-lactams in wastewater, and reusability studies (PDF)

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Notes

The authors declare no competing financial interest.

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