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Real-time measurement of small molecules directly in awake, ambulatory animals

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The development of a technology capable of tracking the levels of drugs, metabolites, and biomarkers in the body continuously and in real time would advance our understanding of health and our ability to detect and treat disease. It would, for example, enable therapies guided by high-resolution, patient-specific pharmacokinetics (including feedback-controlled drug delivery), opening new dimensions in personalized medicine. In response, we demonstrate here the ability of electrochemical aptamer-based (E-AB) sensors to support continuous, real-time, multihour measurements when emplaced directly in the circulatory systems of living animals. Specifically, we have used E-AB sensors to perform the multihour, real-time measurement of four drugs in the bloodstream of even awake, ambulatory rats, achieving precise molecular measurements at clinically relevant detection limits and high (3 s) temporal resolution, attributes suggesting that the approach could provide an important window into the study of physiology and pharmacokinetics.

aptamer | square-wave voltammetry | in vivo | E-DNA | precision medicine

The availability of versatile and convenient sensors supporting the continuous, real-time measurement of specific molecules directly in the body could prove transformative in research and in medicine. In the short term, for example, such an advance would allow the in vivo concentrations of drugs, metabolites, hormones, and other biomarkers to be measured with high precision in subjects as they undergo their normal daily routine, improving our knowledge of physiology, pharmacokinetics, and toxicology. On longer timescales, such an advance would facilitate "therapeutic drug monitoring," in which dosing is personalized using a patient's directly measured (rather than crudely and indirectly estimated) metabolism. By permitting the continuous monitoring of biomarkers (e.g., creatinine and hormones), such a technology would likewise provide a new and highly detailed window into health status (e.g., kidney or endocrine function). Finally, the real-time measurement of specific molecules in the body would advance drug delivery (1). Such a technology, for example, could easily support feedback-controlled dosing, in which the delivery of drugs is adjusted in real time based on their concentration in the body or on the body's molecular-level response to treatment. This real-time, feedback-controlled drug delivery would provide new routes by which drugs with dangerously narrow therapeutic windows or complex optimal dosing regimens can be administered safely and efficiently.

Although technologies already exist for the continuous or near-continuous measurement of a small number of metabolites [e.g., glucose (2) and lactate (3)] and neurotransmitters [e.g., dopamine (4, 5), serotonin (6), glutamate (7), and acetylcholine (8)] in vivo, these approaches all rely on the specific chemical reactivities of their targets (e.g., the redox chemistry of the analyte or its ability to be oxidized by a specific enzyme). Because of their dependence on reactivity, these technologies are not generalizable to the detection of many other physiologically or clinically important molecules, and there remains an open, critical need for strategies that support the continuous detection of specific molecules in the body irrespective of their reactivity. Unfortunately, however, serious technical hurdles stand in the way of realizing this goal (9, 10). First, to support continuous measurements, a sensor cannot rely on batch processing, such as wash or separation steps. Second, to support in vivo measurements, a sensor cannot use exogenously added reagents and must remain stable against prolonged exposure to blood or interstitial fluids in vivo. To date, the vast majority of molecular detection strategies have failed to meet one or both of these critical challenges. Chromatography, mass spectrometry, and immunochemistry, for example, are complex, multistep batch processes requiring wash steps, separation steps, and/or sequential reagent additions, hindering their ability to perform continuous measurements. Conversely, whereas biosensors based on surface plasmon resonance (SPR), quartz crystal microbalances (QCM), field-effect transistors (FET), and microcantilevers all support continuous, real-time operation, each fails when challenged in blood (much less in vivo) due to their inability to discriminate between the specific binding of their target and the nonspecific adsorption of proteins and cells (11–14). Here, in contrast, we demonstrate the ability of electrochemical aptamer-based (E-AB) sensors, a sensing platform adaptable to the detection of any of a wide range of molecular targets irrespective of their chemical reactivity, to support continuous, real-time measurements directly within the body.

Significance

The ability to monitor arbitrary molecules directly in living subjects as they undergo their daily routines remains one of the "holy grails" of bioanalytical chemistry. Such a technology would, for example, vastly improve our knowledge of physiology, pharmacokinetics, and toxicology by allowing the high-precision measurement of drugs and metabolites under realistic physiological conditions. Real-time molecular measurements would also provide an unparalleled window into health status (e.g., kidney function) and would facilitate "therapeutic drug monitoring," in which dosing is personalized to the specific metabolism of each individual patient. Finally, the ability to measure molecules in the body in real time would provide unprecedented new routes by which drugs with dangerously narrow therapeutic windows could be safely and efficiently administered.

Author contributions: N.A.-C., P.A.V., T.E.K., and K.W.P. designed research; N.A.-C., J.S., P.A.V., and K.L.P. performed research; N.A.-C., P.A.V., and T.E.K. contributed new reagents/analytic tools; N.A.-C. and J.S. analyzed data; and N.A.-C., J.S., P.A.V., K.L.P., T.E.K., and K.W.P. wrote the paper.

Conflict of interest statement: K.W.P. discloses service on the scientific advisory boards of Diagnostic Biochips Inc., Ilumi Health, and Eccrine Systems. N.A.-C., J.S., and K.W.P. have filed a provisional patent based on the work presented in this paper.

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The generality of E-AB sensors stems from the versatile recognition and signal-transduction properties of aptamers, nucleic acids selected for their ability to bind specific molecular targets (15). Created using well-established in vitro selection methods (16, 17), aptamers can be generated that bind a wide range of analytes (18) and can be rationally reengineered such that they undergo a large-scale conformational change upon binding these analytes (19) over arbitrarily broad (20, 21) or narrow (20, 22) concentration windows. E-AB sensors use this conformational change to generate an easily measureable electrochemical signal without the need for the target to undergo a chemical transformation (23). To achieve this signal transduction, the aptamer's binding-induced conformational change is used to alter the efficiency with which a covalently attached redox reporter (here methylene blue) approaches an underlying electrode, producing a target-concentration-dependent change in current when the sensor is interrogated using square wave voltammetry (24) (Fig. 1*A* and *SI Appendix*, Fig. S1). As required to support continuous in vivo measurements, E-AB signaling is not reliant on batch processes, such as wash steps, or on the addition of exogenous reagents. Furthermore, because E-AB signaling is generated by a specific, binding-induced conformational change—and not adsorption of the target to the sensor surface (which is the case for SPR, QCM, FETs, and microcantilevers)—the platform is relatively insensitive to fouling. Previous studies, for example, have shown that E-AB sensors perform well when challenged for hours in flowing, undiluted blood serum (25), rendering them one of the most fouling resistant single-step biosensor platforms reported to date.

Despite their unprecedented ability to perform continuous monitoring in undiluted blood serum, first-generation E-AB sensors nevertheless foul when challenged in undiluted whole blood, precluding their use directly in vivo. In response, we previously developed a microfluidic approach to preventing fouling by blood cells that supports continuous ex vivo measurements of drug levels in blood continuously drawn by catheter from anesthetized animals (26). In that work, we constructed a microfluidic device using two stacked laminar flows: a bottom flow of blood continuously drawn via a jugular catheter from the animal and draining into a waste chamber, and a flow of buffer stacked on top of this first layer and in permanent contact with the relevant E-AB sensor. The buffer sheath acts as a continuous-flow diffusion filter, allowing for rapid diffusion of small-molecule targets to the sensor while preventing the approach of (much more slowly diffusing) blood cells. Using this device, we have measured the serum levels of multiple drugs

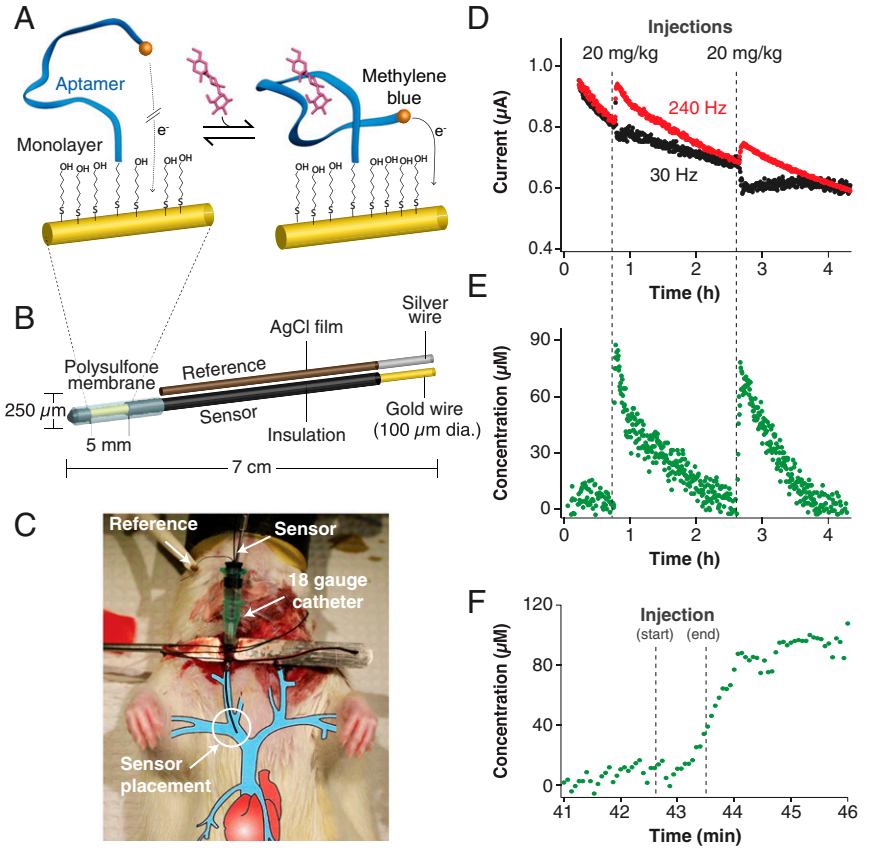

Fig. 1. Real-time, continuous measurement of specific drugs directly in the living body. (*A*) The E-AB sensing platform, in which the binding-induced folding of an electrode-bound, redox-reporter-modified aptamer leads to a change in electron transfer easily detected using square wave voltammetry. (*B*) A microporous (0.2 μm) polysulfone membrane protects the sensor from fouling by blood cells. (*C*) The resultant device is small enough to emplace in one of the external jugulars of a rat using an 18-gauge catheter (the cartoon overlay illustrates sensor location). (*D*) To correct the drift seen in vivo, we record data at two square wave frequencies (here 30 and 240 Hz; optimal values depend on the aptamer used). At one frequency, the sensor's voltammetric signal increases upon target binding, whereas at the other, it is reduced; taking the difference between the two eliminates drift and enhances signal-to-noise (26). (*E*) Using drift-corrected E-AB sensors, we have monitored the in vivo concentrations of multiple drugs continuously and in real-time over the course of many hours in measurements that achieve clinically relevant precision and few-second time resolution. Shown here, for example, is the measurement of the antibiotic tobramycin in the blood of an anesthetized rat after two serial injections into the opposite external jugular. (*F*) At 3 s per measurement, the time resolution of these measurements is sufficient to monitor both the injection itself and the subsequent distribution of the drug within the circulatory system and reflects an orders of magnitude improvement over the resolution of traditional pharmacokinetic methods (45).

for up to 4 h. The approach, however, is nevertheless not without potentially significant limitations. Being ex vivo, for example, the device suffers from a time lag (the time required for blood to leave the body and enter the device), requires continuous blood draws (the buffer-diluted blood must be discarded), and can only be used to measure molecules in blood because other bodily fluids cannot easily and continuously be withdrawn. The device is also complex, requiring a pump and buffer and waste reserves. Finally, due to the necessity of generating laminar flow, the device is sensitive to mechanical shock and thus likely not robust enough to be deployed in awake, freely moving animals. Here, in contrast, we have adapted E-AB sensors to the important problem of performing continuous, real-time, multihour measurements of specific molecules directly within the bodies of awake, freely moving animals.

Results and Discussion

We have taken a two-pronged approach to circumventing the challenging conditions associated with deploying sensors directly within the bodies of living animals. To reduce fouling, we encase our sensors in biocompatible (27) polysulfone membranes (Fig. 1*B*), the 0.2-μm pores of which prevent blood cells from approaching the sensor surface while simultaneously allowing for the rapid transport of target molecules. Using these membranes, we achieve stable E-AB baselines in flowing, undiluted whole blood in vitro over many hours (*SI Appendix*, Fig. S1). Even membrane-protected E-AB sensors, however, exhibit significant baseline drift when emplaced in the veins of live animals (Fig. 1*D*). To circumvent this drift, we use a correction scheme termed "Kinetic Differential Measurements" (KDM). Drift correction methods have historically used a physically separate reference that, although unresponsive to the targeted input, nevertheless yields an identical response to background that can be subtracted from the sensor output (28). KDM instead employs a single aptamer in both roles, thus obviating the need to fabricate a matched sensor-reference pair (26). To achieve this stand-alone performance, KDM exploits the square wave frequency dependence of E-AB signaling. Specifically, electron transfer is more rapid from the folded, target-bound aptamer than it is from the unfolded, target-free aptamer. This kinetic difference results in a binding-induced increase in current when we perform square-wave voltammetry at high frequencies and a binding-induced decrease in signal at low frequencies (*SI Appendix*, Fig. S2) (19). Conveniently, these two outputs drift in concert, and thus taking their difference effectively corrects baseline drift (Fig. 1*D*).

Drift-corrected, membrane-protected E-AB sensors readily support the continuous, seconds-resolved real-time measurement of specific molecules in the blood of living animals (Fig. 1 *E* and *F*). To demonstrate this ability, we first emplaced E-AB sensors for the detection of the cancer chemotherapeutic doxorubicin (DOX) (29, 30) in the external jugular vein of anesthetized Sprague–Dawley rats (Fig. 1*C*). Using this approach, we achieve nanomolar precision in the measurement of clinically relevant plasma drug levels following five sequential injections over 5 h of continuous monitoring (Fig. 2*A*). The resulting plot of concentration versus time presents consecutive spikes corresponding to each of the injections performed, with maximum DOX concentrations (C_{max}) of ~600 nM and the effective clearance of 90% of the drug from the circulatory system within 50 min, values in close accord with prior reports (31).

Because E-AB signaling is independent of the chemical reactivity of the target, E-AB sensors can be switched to the

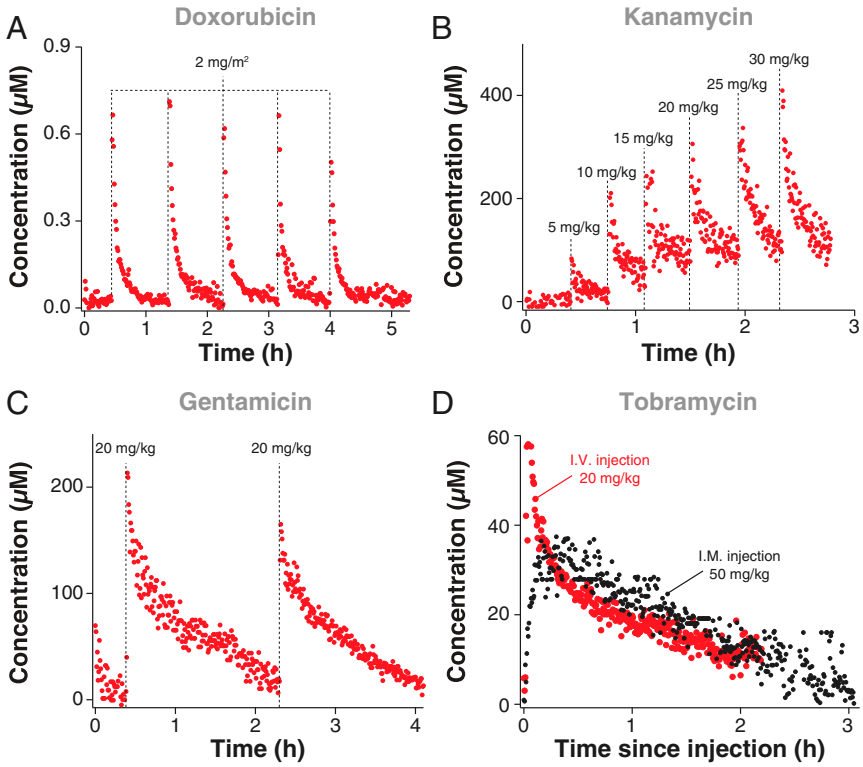

Fig. 2. Continuous molecular measurements in vivo. We have successfully measured multiple drugs using E-AB sensors emplaced in the jugulars of anesthetized rats. (*A*) Shown here are five i.v. injections of 2 mg/m^2 of the cancer chemotherapeutic DOX, a dose more than 25 times lower than typical human doses (46). (*B–D*) To illustrate the generality of the approach, we have also used an aminoglycoside-detecting E-AB sensor to monitor in vivo levels of the antibiotics kanamycin, gentamicin, and tobramycin at the indicated doses. The kanamycin doses used here span the 10–30 mg/kg therapeutic range used in humans (34). For gentamicin, we focus here on two sequential i.v. injections of the drug, separated by a 2-h interval. For tobramycin, we present an overlay of data collected after sequential i.m. (thigh) and i.v. (the external jugular opposite the sensor) injections carried out in a single rat.

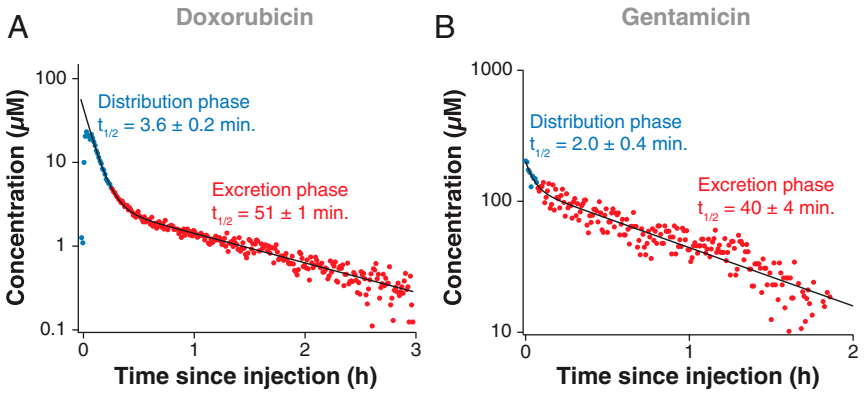

Fig. 3. High-precision pharmacokinetics. Shown are high-resolution pharmacokinetic profiles for the drugs DOX (*A*) and gentamicin (*B*) upon i.v. injection of 50 mg/m^2 and 20 mg/kg doses, respectively. As is easily seen, the resolution of in vivo E-AB sensors is sufficient not only to define the slower β phases of these drugs (red dots) but also to define their much more rapid α phases (blue dots) with excellent statistical significance. These measurements constitute a precise determination of the i.v. distribution phase of a small-molecule drug.

detection of new, chemically unrelated molecules via the simple expedient of replacing their aptamer recognition element. To demonstrate this modularity, we fabricated sensors using an aptamer recognizing the aminoglycoside antibiotics (32, 33). Using these sensors, we first followed monotonically increasing i.v. doses of kanamycin spanning the therapeutic ranges used in humans (34) (10–30 mg/kg) and animals (35) (25–30 mg/kg). The sensor responded rapidly to each injection, measuring maximum concentrations between 34 and 400 μM depending on the delivered dose (Fig. 2*B*). The 200 μM maximum concentration observed after a 10 mg/kg dose was in agreement with peak plasma concentrations determined previously (using cumbersome, poorly time-resolved ex vivo radioimmunoassays) after similar doses were injected into multiple animal species (36). The sensor can likewise monitor in real time the in vivo concentrations of the aminoglycosides gentamycin (Fig. 2*C*) and tobramycin (Fig. 2*D* and *SI Appendix*, Fig. S3) following either i.m. or i.v. injections, applications in which it once again achieves excellent precision and time resolution.

The ability to perform the continuous measurement of specific molecules in the body opens the door to many potentially transformative applications in the study of physiology and pharmacokinetics. For example, the few-second time resolution of E-AB sensors (Fig. 1*F*), which reflects orders of magnitude improvement over the time resolution of traditional pharmacokinetic methods, is sufficient to measure the kinetics with which drugs distribute following i.v. injection (Fig. 3 and *SI Appendix*, Fig. S4), a pharmacokinetic phase that has rarely if ever been previously measured (e.g., refs. 37–39). Indeed, the precision of E-AB measurements is sufficient not only to robustly identify animal-to-animal pharmacokinetic variability, but even variability within a single animal over the course of a few hours. To explore this ability, we monitored the pharmacokinetics of tobramycin following sequential 20 mg/kg i.v. injections conducted 2 h apart in each of three rats. Fitting the resultant data to a two-compartment model, we easily observe statistically significant inter- and even intraanimal variability (Fig. 4). The distribution phase (α phase) of this drug, for example, is defined largely by blood and body volume and thus, although the distribution differs between animals, it differs much less as a function of time within individual animals. The elimination kinetics of tobramycin (β phase), in contrast, not only vary significantly between animals but also exhibit variations within a single individual over the course of a few hours that are easily measurable using our approach (Table 1). For example, although the kinetics of the α phase remain relatively constant for a given animal, the β phase invariably slows with time. This change presumably occurs because, whereas drug absorption (captured by the α phase) is defined by body volume, which remains fixed, the elimination of tobramycin (captured in the β phase) is predominantly via excretion from the kidneys (40, 41), the function of which likely changes due to alterations in the animal's blood pressure (42) and/or hydration after several hours under anesthesia.

The ability of E-AB sensors to reject false signals arising from background interferents is excellent; none of the many

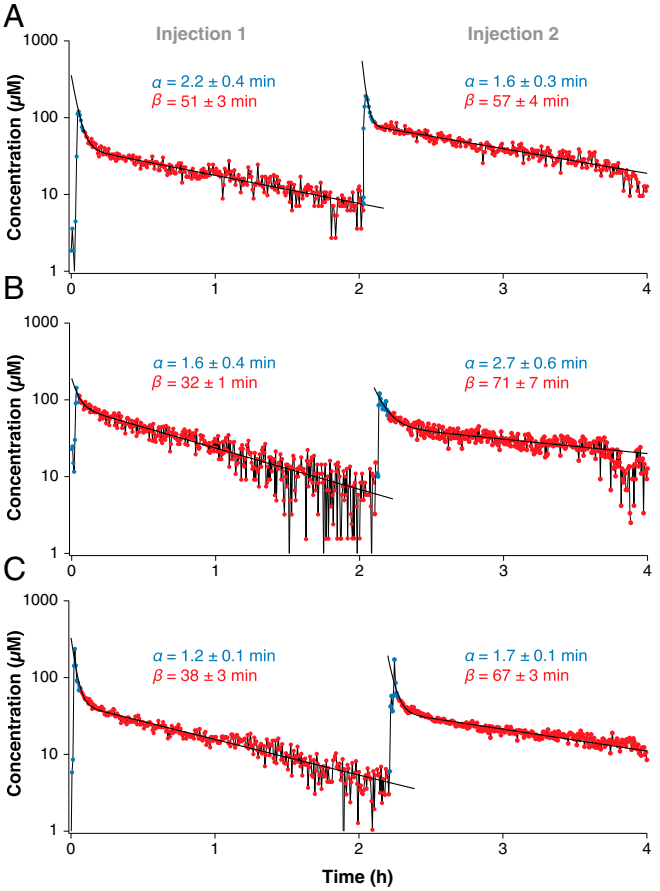

Fig. 4. The measurement of inter- and intraanimal pharmacokinetic variability. The precision of E-AB measurements is sufficient to measure not only interanimal pharmacokinetic variability but also variability within an individual animal over time. Shown are the pharmacokinetic profiles of the drug tobramycin following two sequential 20 mg/kg i.v. injections in three different rats (*A*, *B*, and *C*). These high-precision measurements reveal a decrease in the rate of drug elimination kinetics (β phase) in the second injection with respect to the first in all three animals, an effect that presumably arises due to changes in the animal's blood pressure and/or hydration after several hours under anesthesia. The bold black lines represent the mathematical fit of each injection dataset to a two-compartment pharmacokinetic model.

Table 1. Pharmacokinetic parameters corresponding to repeated i.v. injections of tobramycin in three Sprague–Dawley rats

Rat no.	Injection no.	A,* μM	α, min	B, μM	β, min	C_{max},† μM	AUC, μmol·min·L^{-1}	Cl_T, mL·min^{-1}
1	1	255 ± 82	2.2 ± 0.4	71 ± 2	51 ± 3	326 ± 82	117 ± 58	129 ± 64
1	2	267 ± 56	1.6 ± 0.3	80 ± 4	57 ± 4	347 ± 56	168 ± 61	90 ± 32
2	1	113 ± 38	1.6 ± 0.4	82 ± 2	32 ± 1	195 ± 38	73 ± 39	208 ± 100
2	2	109 ± 22	2.7 ± 0.6	50 ± 2	71 ± 7	159 ± 22	41 ± 15	370 ± 139
3	1	284 ± 34	1.2 ± 0.1	46 ± 2	38 ± 3	330 ± 34	237 ± 49	63 ± 13
3	2	138 ± 32	1.7 ± 0.1	35 ± 2	67 ± 3	173 ± 32	82 ± 28	186 ± 63

Confidence ranges reflect 95% confidence intervals.

*A, α, B, and β are derived from the fit to a two compartment model: $[\text{target}] = Ae^{-t/\alpha} + Be^{-t/\beta}$, where α and β are the half-lives for distribution and elimination, respectively.

†C_{max}, AUC (area under the curve), Cl_T (drug clearance), and their associated confidence intervals propagated from the kinetic parameters A, α, B, and β.

endogenous metabolites and hormones in rat blood activates the sensor, as evidenced by their performance in vivo. The platform's ability to distinguish between structurally similar molecules, in contrast, can be problematic due to the sometimes [although not always (43, 44)] limited specificity of aptamers because, of course, the sensor cannot be more specific than the aptamer from which it is constructed. E-AB specificity is nevertheless sufficient for many research and clinical applications. For example, although the aminoglycoside-binding aptamer recognizes multiple members of this closely related family of drugs (Fig. 2 *B–D*), coadministration of more than one of these highly toxic drugs is clinically contraindicated, and thus the inability to distinguish between them is of little medical relevance. The therapeutic action of DOX is driven by its ability to bind DNA, and thus the aminoglycoside sensor also exhibits cross-reactivity to this drug (*SI Appendix*, Fig. S5*A*). Here too, however, the coadministration of the two is so rare as to limit the clinical impact of this effect. The DOX-detecting sensor, in contrast, exhibits no significant cross-reactivity with the aminoglycosides (*SI Appendix*, Fig. S5*B*), nor does it exhibit significant cross-reactivity with other chemotherapeutics that are commonly coadministered with DOX in clinical applications (26).

In addition to studies, as those above, performed on anesthetized animals, the simplicity, physical robustness, and small size of E-AB sensors also renders it possible to perform measurements on awake, ambulatory animals. To illustrate this ability, we surgically implanted permanent catheters in the jugular veins of rats and allowed the animals to recover from this surgery for 2 wk before using the catheter to insert a flexible E-AB sensor under light anesthesia. The sensor connects to its supporting electronics via flexible wire leads that allow the awake animals to move largely unimpeded (Fig. 5*A* and Movie S1). Aminoglycoside sensors used under these conditions support run times of up to half a day as the animal feeds, drinks, and explores its environment (Fig. 5 *B* and *C*), producing pharmacokinetic data that avoid potentially confounding factors associated with measurements based on (repeated) blood draws, which require anesthetized or otherwise immobilized (and thus stressed) animals.

Here, we demonstrate the ability of E-AB sensors to track specific small molecules continuously and in real time in awake,

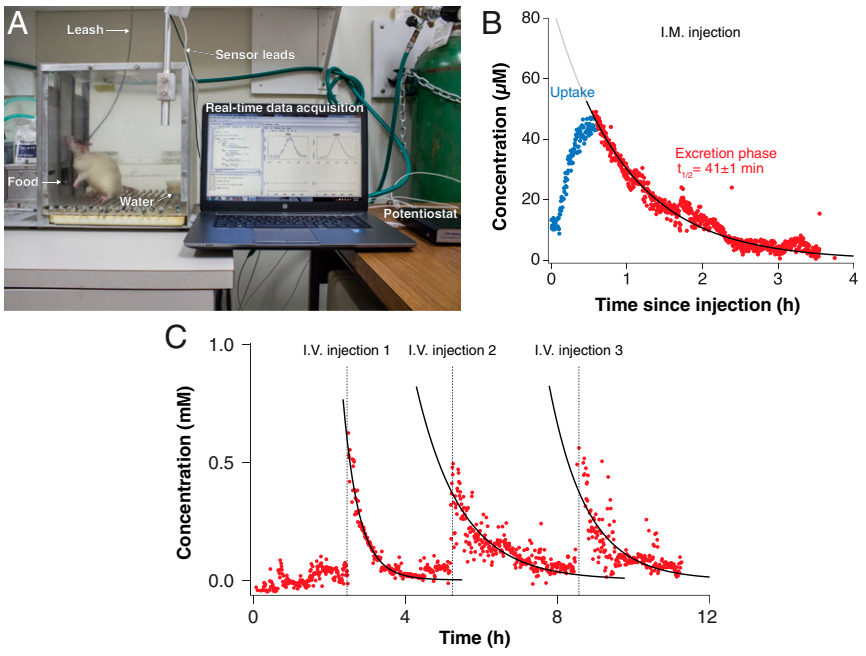

Fig. 5. Continuous, in vivo molecular measurements on awake, ambulatory animals. (*A*) The small size and physical robustness of E-AB sensors renders it possible to use them in animals as they eat, drink, and explore their cage (Movie S1). This robustness, in turn, enables the measurement of specific molecules in the blood of animals as they undertake their normal daily routine, conditions perhaps more relevant to human health than those traditionally used for the collection of metabolic and pharmacokinetic data. Shown are blood levels of the drug tobramycin after a 25 mg/kg i.m. injection (thigh) (*B*) or sequential 40 mg/kg i.v. (jugular vein) injections (*C*) in awake, freely moving animals.

ambulatory animals, a capability that could provide an important tool for understanding physiology and pharmacology. By allowing arbitrary molecules (limited only by the availability of an aptamer of appropriate specificity and affinity) to be monitored with high resolution in animals undergoing their normal daily routine, for example, the ability to perform such measurements could improve our knowledge of metabolism, pharmacokinetics, and toxicology. The few-second time resolution of our approach likewise suggests that it could improve our understanding of rapidly fluctuating physiological events, such as uptake and distribution pharmacokinetics, hormone and neurotransmitter release, and the movement of drugs and metabolites across the blood–brain barrier and within the central nervous system. Finally, the ability to perform the measurement of specific molecules in the body in real time could enhance the efficiency and accuracy with which drugs are dosed, in applications ranging from personalized, patient-specific pharmacokinetic measurements as a means of precisely tailoring dosing to long-term feedback-controlled drug delivery in which the dosage of a drug is varied in real time in response to minute-to-minute changes in a patient's physiological status. In short, the technology demonstrated here could enhance not only our understanding of health but also our ability to detect, monitor, and treat disease.

Materials and Methods

All animal procedures were consistent with the guidelines of the NIH *Guide for the Care and Use of Laboratory Animals* (47) and approved by the Institutional Animal Care and Use Committee of the University of California, Santa Barbara.

A detailed description of the materials and methods used in this work can be found in *SI Appendix*, which includes a descriptive list of chemicals and materials used, E-AB sensor fabrication, calibration and surgical emplacement, a description of the electrochemical methods (including KDM) and data analysis software used, and details on the experimental setups used to carry out in vitro and in vivo measurements.

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