



Published in final edited form as:

J Sci Med Sport. 2023 June ; 26(Suppl 1): S46–S53. doi:10.1016/j.jsams.2023.01.010.

Efforts toward the continuous monitoring of molecular markers of performance

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Abstract

Objectives: Technologies supporting the continuous, real-time measurement of blood oxygen saturation and plasma glucose levels have improved our ability to monitor performance status. Our ability to monitor other molecular markers of performance, however, including the hormones known to indicate overtraining and general health, has lagged. That is, although a number of other molecular markers of performance status have been identified, we have struggled to develop viable technologies supporting their real-time monitoring in the body. Here we review biosensor approaches that may support such measurements, as well as the molecules potentially of greatest interest to monitor.

Design: Narrative literature review.

Method: Literature review.

Results: Significant effort has been made to harness the specificity, affinity, and generalizability of biomolecular recognition in a platform technology supporting continuous in vivo molecular measurements. Most biosensor approaches, however, are either not generalizable to most targets, or fail when challenged in the complex environments found in vivo. Electrochemical aptamer-

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Declaration of interest statement

KWP and RHB own equity in and RHB is an employee of a company seeking to commercialize EAB sensors for applications in vivo molecular monitoring. JH also owns equity in and is an employee of a company seeking to commercialize EAB sensors for applications in vivo molecular monitoring.

based sensors, in contrast, are the first technology to simultaneously achieve both of these critical attributes. In an effort to illustrate the potential of this platform technology, we both critically review the literature describing it and briefly survey some of the molecular performance markers we believe will prove advantageous to monitor using it.

Conclusions: Electrochemical aptamer-based sensors may be the first truly generalizable technology for monitoring specific molecules in situ in the body and how adaptation of the platform to subcutaneous microneedles will enable the real-time monitoring of performance markers via a wearable, minimally invasive device

Keywords

Wearable electronic devices; Electrochemistry; Technology; Aptamers; Pharmacokinetics; Health monitoring

1. Introduction

Although few in number, the technologies supporting real-time molecular measurements in the body have significantly impacted our ability to monitor human performance status. The pulse oximeter, for example, which reports changes in blood oxygen levels in real-time, has rapidly expanded from a specialty surgical technology to a ubiquitous clinical and performance-monitoring tool.¹ By providing real-time information on blood sugar levels, wearable continuous glucose monitors have similarly revolutionized the treatment of diabetes, and are making rapid inroads into performance monitoring.^{2,3} Beyond oxygen and glucose, however, our ability to monitor specific molecules in the body in real time remains limited. For example, while in January 2022, medical device provider Abbott Laboratories announced their wearable biosensor Lingo product line to continuously monitor ketones, lactate, alcohol, and glucose, these devices are not yet commercially available.⁴ Beyond this, until recently, only a handful of other molecules have been measured in real-time in vivo, even just as laboratory demonstrations (e.g., glutamate, acetylcholine).^{5–7}

The glucose monitor and the pulse oximeter are biosensors, a class of technologies that has long been promised to be general, but that has yet to realize this vision in in vivo applications. The problem is, like all previous approaches to in vivo molecular monitoring, the glucose monitor and the pulse oximeter rely critically on the chemical reactivity of their targets. Specifically, glucose sensors use glucose oxidase to convert its target into an easily measurable product (hydrogen peroxide), and the pulse oximeter monitors the color change that occurs when oxygen binds to hemoglobin.^{1,8} And, although we can now generate proteins and nucleic acids that will recognize almost any water-soluble target,⁹ we cannot generate peroxide-producing enzymes or color-changing receptors at will, thus greatly limiting the extent to which sensing approaches analogous to the glucose meter or the pulse oximeter can be expanded to new targets. In short, achieving the goal of a generalizable ability to monitor specific molecules in the body will require the development of technologies that are independent of the chemical reactivity of their targets.

For many years, the primary focus of efforts to build biosensor “platforms” that are independent of the reactivity of their targets has been “adsorption-based” biosensors that

rely on target detection via changes in mass, charge, steric bulk or optical properties associated with the adsorption of the target to a biomolecular receptor attached to a surface. Examples of this include sensors based on surface plasmon resonance, field effect transistors, quartz crystal microbalances, and microcantilevers.^{10,11} Unfortunately, however, when challenged in bodily fluids these approaches are overwhelmed by the non-specific adsorption of proteins, which produces signals that are indistinguishable from those produced by authentic target binding.^{12,13}

In contrast to most biosensor architectures, the chemoperception systems found in nature are both selective enough to work in vivo and independent of the chemical reactivity of their targets.¹⁴ To achieve both of these attributes simultaneously, evolution designed the relevant receptors such that they undergo a large-scale conformational change upon target binding.¹⁵ This, in turn, generates an output that is not mimicked by non-specific adsorption. Inspired by this naturally occurring phenomenon, a new class of artificial biosensors, termed electrochemical, aptamer-based (EAB) sensors, has recently achieved the breakthrough milestone of monitoring molecules in vivo without relying on their reactivity (Fig. 1).^{16–22} In this review, we first describe EAB sensors before then surveying in vivo EAB sensor literature. Finally, we close by discussing the adaptation of EAB sensors to performance monitoring.

2. Electrochemical aptamer-based sensors

In addition to achieving physiologically relevant sensitivity and specificity, an ideal platform technology supporting continuous, real-time in vivo molecular monitoring must achieve all of the following attributes:

1. Generalizable: i.e., independent of the chemical reactivity of its targets
2. Selective: it must perform well in complex bodily fluids
3. Reversible: it must reverse on a timescale that is rapid relative to the relevant physiology
4. Stable: it must remain drift-free in the complex, time-varying environments found in vivo.

Over the last five years, EAB sensors have, through the application of several technological advances, achieved all of these critical attributes.

The first advance of the EAB platform relies on the employment of aptamers as their recognition element. Aptamers are synthetic oligonucleotides produced by an in vitro selection process (Fig. 2) that starts with a random library of oligonucleotide sequences.^{9,21} These sequences are incubated with the desired target, any non-binding sequences are removed, and the bound sequences are amplified and cycled through the same process again, ultimately leading to a pool of high-performance receptors.^{9,23} The use of such sequences as the recognition element in EAB sensors renders the approach highly modular. Consistent with this, EAB sensors have been reported for the detection of dozens of different targets, including a wide range of drugs,^{16,24–29} metabolites,^{17,30} hormones,^{31–33} and protein biomarkers.^{34–37}

A second, critical advance is the use of binding-induced conformational change to transduce binding into an electrochemical output, a marriage that renders EAB sensors independent of the reactivity of their targets, rapidly reversible, and selective enough to deploy in bodily fluids. Several approaches have been described to date by which allow rational re-engineering of binding-induced conformational changes into aptamers suitable for the EAB platform.³⁸ Most commonly, this is achieved by destabilizing the aptamer either through changing G-C base pairs to A-T base pairs to decrease the melting temperature of predicted internal double-stranded regions or truncation.^{19,39,40} Truncation can be accomplished either by simply shortening the predicted double-stranded regions or using exonuclease digestion to identify the minimum aptamer sequence required for target binding.^{41,42} With either re-engineering method, the aptamer thus equilibrates between an unfolded state, which does not bind the target, and the folded state, which does. As target binding drives the equilibrium toward the folded state, a very large conformational change occurs.

The selectivity of EAB sensors depends not only on their use of binding-induced conformational changes as a means of signal transduction, but also on the mechanism that they use to monitor this conformational change. Historically, bioengineers have most often used optical approaches to monitor conformational changes such as the binding-induced conformational change underlying EAB sensors.^{43,44} Unfortunately, however, due to the absorbance, scattering, and autofluorescence associated with cells and biomolecules the signal-to-noise of optical sensors suffers when they are deployed in biological fluids.⁴⁵ In contrast, the electrochemical methods used in the continuous glucose monitor work far better in vivo, motivating the development of electrochemical methods for monitoring binding-induced conformational changes.⁴⁶ To achieve this, we covalently link a redox-reporter (such as methylene blue) to one end of the biomolecule, and then attach the other to an interrogating electrode (via thiol-gold self-assembled monolayer chemistry). Any binding-induced change in the shape of the biomolecule alters the efficiency with which the reporter approaches the electrode, changing the rate of electron transfer (Fig. 3A) in a manner that is easily monitored electrochemically (Fig. 3B).

A third advance, drift correction, was the last hurdle necessary to enable the in vivo deployment of EAB sensors. Conformation-linked signaling, which mimics the signal transduction employed by chemoperception systems in the body, is surprisingly resistant to the effects of non-specific adsorption in undiluted blood serum.⁴⁷ Nevertheless, when challenged in situ in the body, EAB sensors exhibit significant drift, likely a result of fouling of the gold working electrode surface.⁴⁸ Fortunately, we developed several methods to correct or eliminate drift. Of these, the most widely used is kinetic differential measurement (KDM), which employs paired square wave frequencies at which the sensor signal drifts in concert but at which its response to target varies dramatically (Fig. 3C and D).⁴⁹ More recently, we have also shown that the use of chronoamperometry for sensor interrogation^{50,51} or the deployment of fouling-resistant coatings on the sensor surface^{16,52} largely or entirely alleviates in vivo drift.

3. In vivo EAB sensors

The EAB platform is the first molecular measurement technology that is both independent of the reactivity of its targets and performs well when challenged in the living body. In the first report of in vivo EAB sensors, for example, we employed KDM-corrected, 75- μ m-diameter, 3-mm-long sensors placed in the jugular veins of live rats, where they were used to monitor the plasma concentrations of four different drugs (doxorubicin, tobramycin, gentamycin, kanamycin) with few-second time resolution.¹⁶ These measurements, which were performed in both anesthetized and awake, freely moving animals, extended for up to 12 h. Building on this, we and others have since expanded the list of molecular targets measured using intravenous EAB sensors to a range of chemotherapeutics, antibiotics, drugs of abuse, and metabolites (Fig. 1), including irinotecan,²⁶ vancomycin,¹⁹ ATP,⁵³ and phenylalanine.¹⁷ In each case, the resulting in vivo measurements achieve few-second to sub-second resolution and clinically and physiologically-relevant measurement precision over the course of hours.

While the above-described studies all employed intravenous sensors, more recent work has demonstrated real-time molecular measurements in the interstitial fluid (ISF) of solid tissues.^{22,24,54} Taylor et al., for example, demonstrated the measurement of cocaine concentrations in the brains of rats.²⁴ Likewise, using a microelectrode array EAB sensor, Seo et al. spatially (and temporally) resolved doxorubicin levels across a tumor (Fig. 1F).²² Most recently, Wu et al. used an EAB sensor-based microneedle array to multiplex irinotecan and doxorubicin monitoring in the dermal ISF of rats.⁵⁴

4. Toward EAB sensors for the monitoring of performance markers

Although already on a strong footing, several steps remain before EAB sensors can be adapted to the routine monitoring of performance markers. In this section, we outline the remaining technological and scientific hurdles.

The intravenous placements used in most in vivo EAB sensor demonstrations to date are presumably too invasive to support routine performance monitoring, which is better suited by subcutaneous placements, such as those employed for the continuous glucose monitor. Achieving these minimally invasive sensor placements will require two advances, both of which are ongoing. First, the EAB platform must be adapted to needles that can be placed into the subcutaneous space. Second, we must improve our understanding of how molecular concentrations in the subcutaneous ISF relate to the molecular concentrations found in systemic circulation.

Microneedles were first studied in 1976, but they did not see widespread use until the late 1990s, when advances in microfabrication technology lead to improved microneedle production with materials such as silicon, glass, ceramics, metals, polymers, and carbohydrates.^{55,56} To date, microneedles have been widely applied to transdermal delivery of therapeutic compounds (e.g., insulin, vaccines),^{57,58} but more recently they have been applied to monitoring disease markers in the subcutaneous ISF.⁸ Recently, microneedle EAB-based sensor arrays detected small molecules in the subcutaneous ISF of rats, suggesting that adaptation of the EAB platform is viable.^{54,59} The microneedle arrays vary

in structure and range from solid needles on which the receptor is attached to hollow needles in which the receptor lines the lumen.⁶⁰ These microneedle-arrays have been used in enzyme-based electrochemical biosensors like the continuous glucose monitor, which suggests that our EAB sensor technology can be readily adapted to an array-type platform.^{8,61} This said, concerns remain for microneedles, including unreliable insertion and the associated potential for signal artifacts in active, ambulatory users. If microneedles are insufficient, however, longer indwelling needles like those currently used in continuous glucose meters are likely a viable, if more invasive, alternative.⁶²

A second hurdle that must be surmounted before convenient, wearable EAB sensors can be realized is the furthering of our understanding of how the levels of markers in the ISF reflects their concentrations in the plasma. Fortunately, while these relationships can be complex, ample literature suggests that the monitoring of biomarkers in the ISF, urine, saliva, and sweat can serve as indicators of health and, presumably, performance.⁶² For example, cortisol, a hormone indicator of stress, can be measured in both the saliva and blood, with the two being strongly correlated.⁶³ Subcutaneous glucose levels parallel plasma glucose levels with time delay of 5 to 12 min and error of less than 6%,² and vancomycin levels measured in extracted ISF peak ~ 17 min later than plasma levels, but the overall concentrations do not differ significantly.⁶⁴ The delays such as these, as well as potential differences in the equilibrium ISF and plasma concentrations, will likely vary with target lipophilicity, charge, and molecular weight.⁶² Nevertheless, we believe the good concordances seen for the above-described targets suggest that the routine monitoring of molecular performance markers using subcutaneous EAB sensors is quite plausible.

5. The performance markers of potential interest

Now that continuous, real-time molecular measurements are on the horizon, the need to identify the molecules for which such monitoring would provide a significantly improved window into performance status is at hand. To some extent, this is a “chicken or egg” problem; without the ability to monitor the candidate molecules continuously and in real time, it is difficult to prove which would prove the most informative when so monitored. Nevertheless, we believe that a number of the performance markers that have already been identified are ideal candidates for adaptation to the EAB platform.

To date, we have primarily focused on the markers of cumulative stress and insufficient recovery, psychological maladaptation, and hormone imbalances, as each of these states leads to decreased physical and cognitive performance and increased risk of injury.^{65,66} The molecular systems most relevant to monitoring stress and recovery are the hypothalamic–pituitary–adrenal (HPA) axis, a key stress response system critical to energy mobilization, and hypothalamic–pituitary–gonadal (HPG) axis, which regulates reproduction as well as muscle growth and recovery (Fig. 4). Each of these axes coordinates inter-organ and tissue crosstalk via neuroendocrine means that are regulated by negative feedback mechanisms, with the two working both in parallel and in conjunction to minimize cumulative stress and promote survival.⁶⁷ Dysregulation of the HPA and HPG axes, for example, is known to contribute to many of the symptoms that comprise overtraining, functional over-reaching, and non-functional overreaching syndromes.⁶⁸

The HPA axis, the dysregulation of which is associated with both physical (e.g., type II diabetes) and mental health disorders (e.g., depression),⁶⁹ is comprised of a hormonal signal cascade that begins with the release of corticotropin releasing hormone (CRH) from the hypothalamus. This stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH) into circulation,⁶⁹ which then causes the zona fasciculata of the adrenal cortex to secrete cortisol. This primary end product of the HPA axis, which is one of the most widely studied biomarkers in stress research,⁷⁰ alters a myriad of physiological functions, including the mobilization and replenishment of energy stores, increased arousal and vigilance, and inhibition of nonessential anabolic activity, which collectively contribute to adaptations during an acute response to stress.^{71,72} Historically, the molecular markers of this axis have proven difficult or impossible to measure with physiologically relevant time resolution. CRH, for example, is released in such small quantities that it is unable to be measured in circulation, and the plasma half-life of ACTH on the order of ~ 4.5 min.⁶⁹ Cortisol, in contrast, has proven more easily measured, with its concentrations typically rising ~ 3 to 5 min after HPA axis activation and peaking ~ 30 min later.⁶⁹ Even the longest of these timescales, however, emphasize the need for the type of convenient, highly-time-resolved measurements enabled by EAB sensing. We note too that, while there are not yet reports of their adaptation into EAB sensors, high-performance cortisol aptamers are available.³³

Like the HPA axis, the HPG axis consists of several hormones released in a cascading manner. Specifically, along this axis gonadotropin releasing hormone (GnRH) is released from the hypothalamus, which stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. LH targets the ovaries and testes to produce estrogens and testosterone, which exert system-wide effects including red blood cell production, glycogen replenishment, bone metabolism, and muscle protein synthesis.⁷³ Monitoring muscle status through measurements of circulating testosterone and LH can help tailor training and inform exercise and recovery intervals.⁷³ In females, circulating estrogen directly influences both energy metabolism and bone mineral density.⁷⁴ Specifically, estrogen increases lipolysis, fat oxidation, and bone strength. Chronic low energy availability (i.e., energy intake minus exercise energy expenditure expressed relative to fat free mass), common in athletes, causes estrogen to decline that induces metabolic disturbances and increased risk of stress fracture.⁷⁵ Here, too, we note that a EAB sensor against LH has been described, and while it has not yet been adapted to in vivo use, it has been used to monitor the hormone ex vivo in small volume blood draws from human subjects.³²

The HPA and HPG axes rely on the rapid, pulsatile release of their constituent hormones, suggesting that seconds-resolved monitoring will provide clear measures of their function and dysregulation. HPA activation, for example, occurs in three distinct temporal patterns: (1) “ultradian” (i.e., between 1 and 24 h) oscillations that occur every 60 min, (2) circadian (i.e., “about a day”) variation decreases as the day progresses, and (3) stress-induced secretion that occurs within minutes of stress onset.⁶⁹ The plasma half-lives of estradiol and testosterone are less than 20 min whereas progesterone has a half-life of approximately 5 min, requiring frequent sampling to capture recurrent, transient changes.^{76,77} Cortisol secretion is also highly variable based on social and emotional experiences, which may account for over 70% of the variation in the cortisol response.^{70,78} On the HPG axis,

variations in GnRH pulse amplitude and frequency similarly govern the synthesis and secretion of LH and FSH, with LH stimulated at high GnRH pulse frequencies (every 60 min or less) and FSH stimulated at low (every 3 h) GnRH pulse frequencies.^{32,79} In fertile females, LH pulsatility varies considerably throughout the menstrual cycle, occurring every 1 to 2 h during the follicular phase (first day of menstruation to ovulation), every 4 h during the luteal phase, and peaks just before ovulation.⁸⁰ For those in menopause or diagnosed with hypothalamic amenorrhea, LH pulsatility becomes quite rapid (~ 50 min),³² albeit it can also be highly disordered, suppressed, or even non-existent.³² Over the course of the day, testosterone concentrations decline 20 to 25% in young males, but only ~ 10% in older males.⁸¹ The many factors that influence the hormonal milieu of these axes, including ultradian (or even more rapid) pulsatility, diurnal variation, age, and menstruation-status, lead to a high degree of variability between individuals (Table 1).^{69,77,82} The highly-time-resolved monitoring of these markers would provide a more accurate assessment of axis integrity and, with that, performance status.

Other factors not detailed in this review should also be taken into consideration for a system-based approach. Notably, binding proteins modulate the amount of free (i.e., active) hormone available to the body and contribute to the total concentration of a hormone in circulation, producing additional challenges to real-time measurements.⁷⁷ Nonetheless, HPA and HPG function have numerous real-world applications and provide a starting point for real-time in vivo monitoring.

6. Conclusions

In this paper we address two questions. The first is, is there a technology on the horizon that might support the continuous, real-time, in vivo monitoring of a wide range of molecular markers in support of performance monitoring? And, although hurdles remain before the approach can be adapted to minimally invasive, easily wearable technology, we believe the answer to this question can be found in the EAB sensor platform. Although the EAB sensor platform appears promising, the majority of reported in vivo EAB sensors only achieve micromolar limits of detection, which is well above the nanomolar levels necessary to meaningfully monitor many hormones. This said, some EAB sensors do achieve nanomolar limits of detection, such as for luteinizing hormone and neutrophil gelatinase-associated lipocalin.^{32,35} Further improvements in the generation of high affinity aptamers, however, will be required to detect clinically relevant levels of many important targets. The second question is, if such a technology were available, would it prove valuable for performance monitoring? Given that dysregulation of the HPA and HPG axes is known to contribute to many of the symptoms that comprise overtraining, functional overreaching, and non-functional overreaching syndromes, and given the rapid timescales over which many of these molecules fluctuate, we believe the answer to this question is also yes. Moreover, we also believe that, once continuous, real-time molecular measurements become available in a platform technology, the resulting, unprecedentedly high-time-resolution data regarding molecular physiology will allow for the identification of many more molecules for which such monitoring will prove of value. In short, we believe a revolutionary new window into performance is on the horizon.

Funding information

Our work (JH, TEK, KWP) in this arena has been supported by grants from the ONR (N00014–20–1–2164 and N00014–20–1–2764) and the NIH (RO1EB022015). CF acknowledges support from an Otis Williams Postdoctoral Fellowship.

Transparency declaration

This article is published as part of a Supplement entitled ‘Macquarie University [MQ] Military Biomarker Symposium 2022’ supported by the Defence Science and Technology Group (DSTG). Funding for the Symposium was provided by Office of Naval Research Global through the Collaborative Science Program. Additional funding was provided by the NSW Defence Innovation Network, Smartabase/Fusion Sport, and Vicon.

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Practical implications

- We identified molecular biomarkers that report on an extensive range of vital information regarding health and performance status.
- Of these biomarkers, only glucose and blood oxygenation sensors are commercially available; our ability to monitor other molecular markers in the body in real time is exceedingly limited.
- Recent advances in biosensors, however, offer the promise of a minimally-invasive platform technology that can monitor a wide range of molecules in the body, in real-time, and with seconds time resolution.
- The electrochemical aptamer-based sensors are a platform technology that will provide an unprecedented window into an individual's molecular physiology, revolutionizing individualized health and performance metrics and personalized recovery plans.

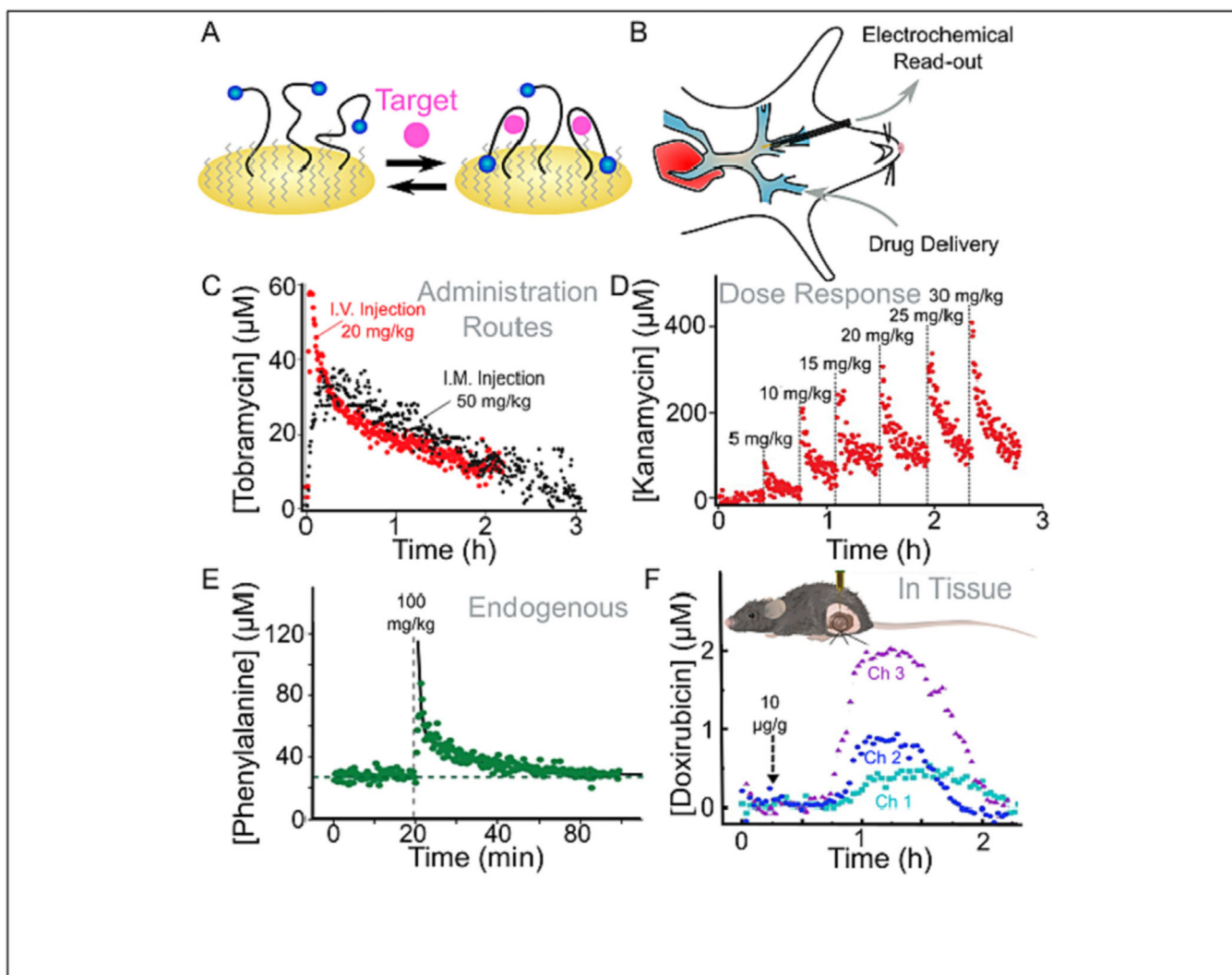


Fig. 1. Electrochemical aptamer-based (EAB) sensors are the first real-time molecular monitoring technology that is both independent of the reactivity of its targets and able to work in the complex, time-varying environments found in the body. (A) EAB sensors consist of an aptamer re-engineered to undergo reversible binding-induced folding. This is modified with a redox reporter and attached (via a self-assembled monolayer) to a gold electrode. The binding-induced conformational change causes a corresponding change in electron transfer rate that is easily monitored using electrochemistry. (B) Bundled with its counter and reference electrodes, current intravenous EAB sensors are small enough and flexible enough to be emplaced, for example, in a rat jugular vein. Using such in vivo EAB sensors, we and others have (C) collected drug pharmacokinetic timecourses¹⁶ and (D) dose response curves,¹⁶ (E) monitored the levels of endogenous metabolites,¹⁷ and (F) spatially resolved drug levels across a tumor.²² Panels C-F are adapted with permission from their respective references.

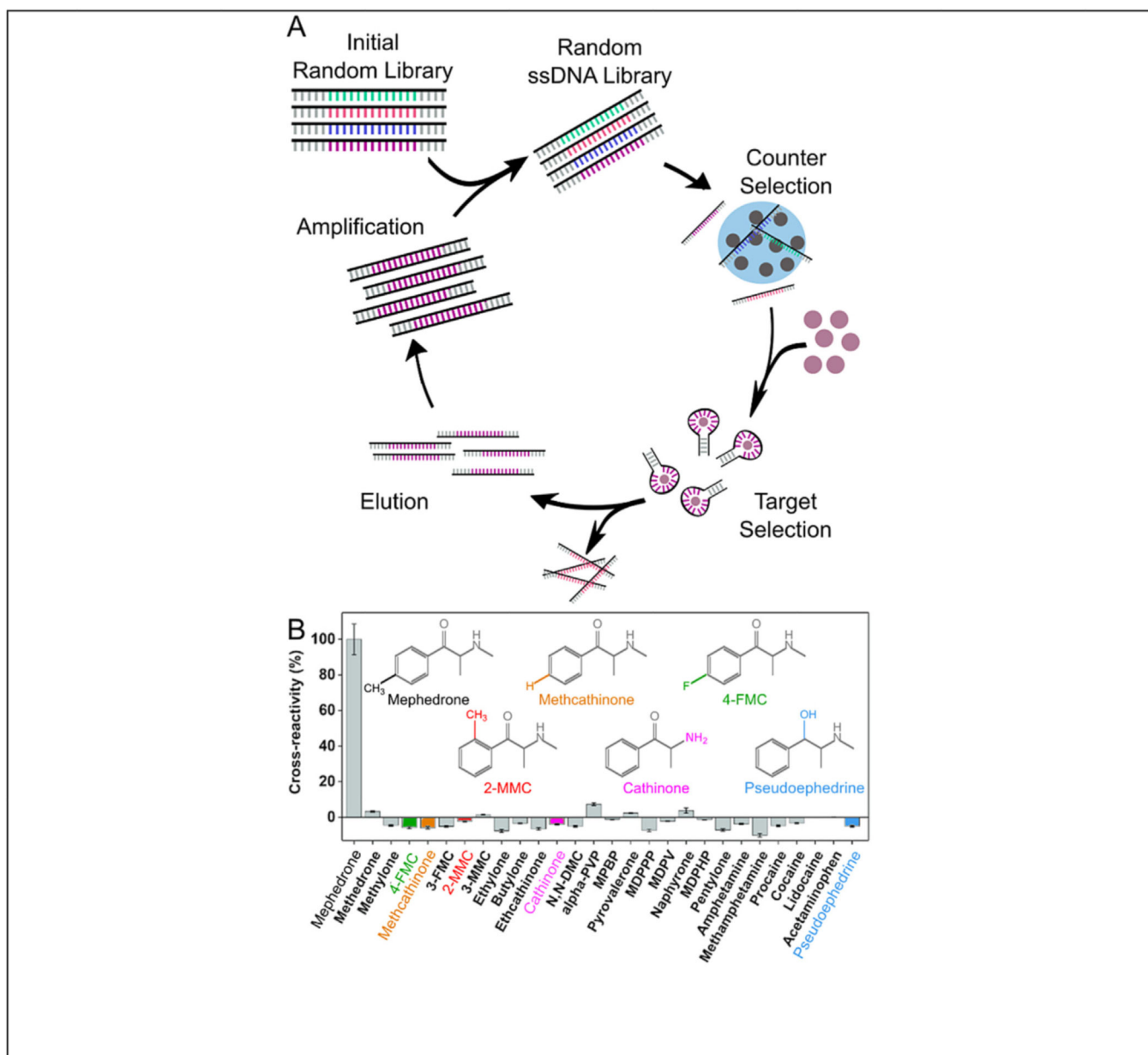
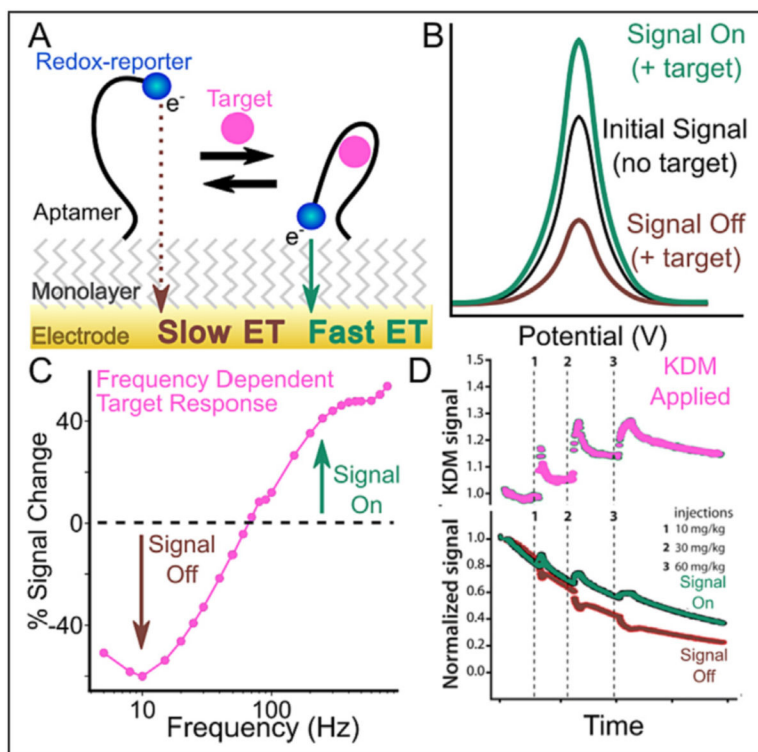


Fig. 2. EAB sensors employ aptamers, synthetic oligonucleotides generated via an in vitro evolutionary scheme, as their recognition elements. (A) In this process, a library of random sequence, single stranded DNAs is introduced to targets of similar structure to the target of interest and any bound sequences are removed to select against sequences of poor specificity. The remaining sequences are then introduced to the target and unbound sequences are removed. The remaining sequences are then amplified and the cycle repeated until it converges on a pool of high affinity, high-specificity receptors. (B) Aptamers can be of exceptional affinity and specificity. Shown, for example, is the cross-reactivity of a mephedrone-binding aptamer with both synthetic and natural cathinone analogs. Adapted with permission from ref 83.

**Fig. 3.**

The strong square-wave frequency dependence of EAB signaling provides routes to improve signal gain and correct the drift seen in vivo. (A) Signal generation in EAB sensors is driven by binding-induced changes in the rate with which an attached redox reporter transfers electrons. (B, C) The resulting change in electron transfer rate is easily monitored using square wave voltammetry, the frequency of which can be tuned such that the sensor's signal increases ("signal on" behavior) or decreases ("signal off") upon target binding.³⁸ (D) To correct for the drift seen in vivo, a "signal on" and "signal off" frequency are selected that drift in concert. Taking the difference between these increases the observed signal and removes the drift.

Adapted with permission from ref 17.

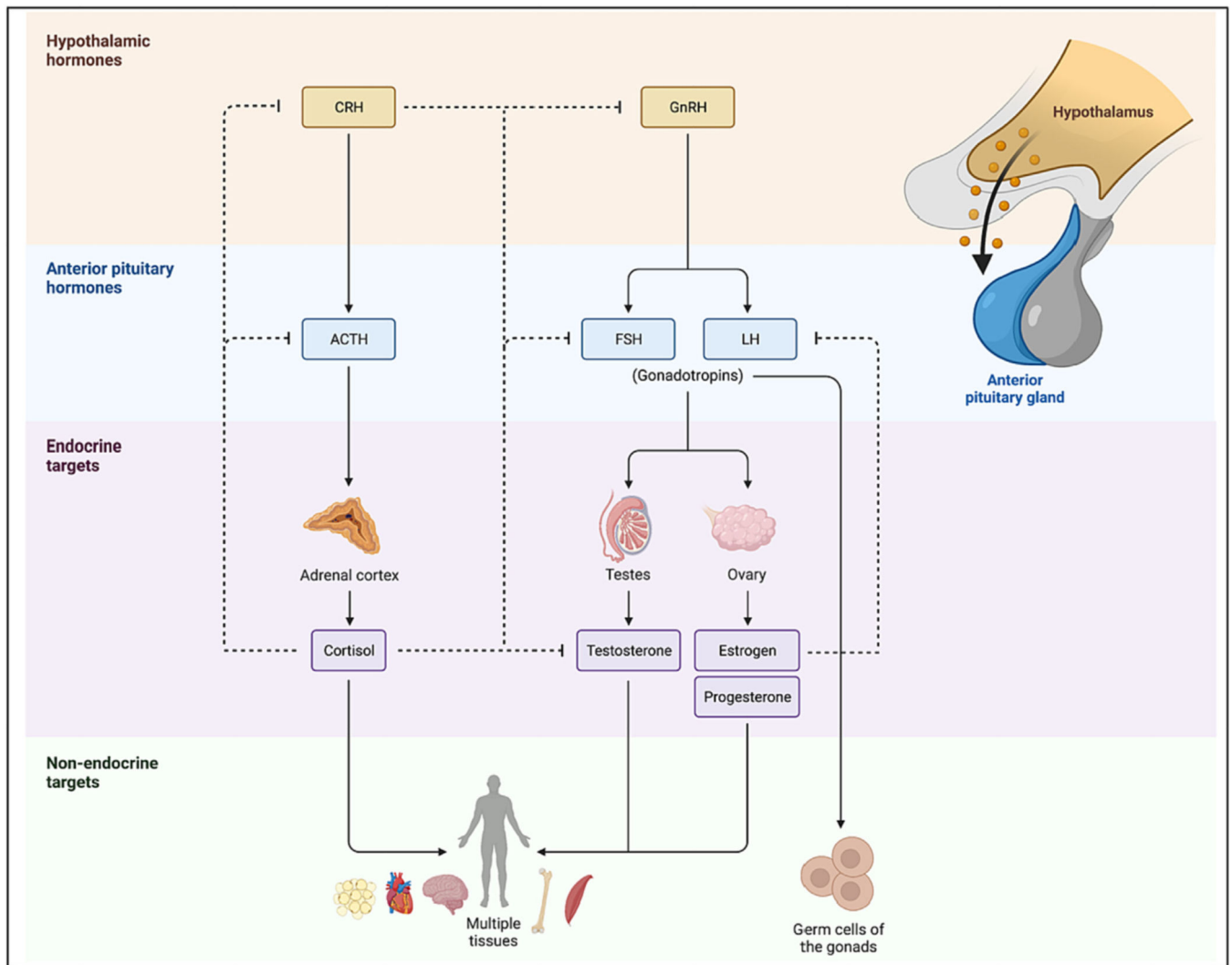


Fig. 4. Overview of the feedback mechanisms involved in the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes. Solid lines represent stimulation whereas dotted lines represent inhibition. Figure created with BioRender.com.

Table 1

Expected hormone ranges in men and women (combined).

Hormone	Concentration range
Adrenocorticotrophic hormone	5.0–80.0 pg/mL ⁶⁹
Cortisol	9.71–554 ng/mL ⁷⁷
Follicle stimulating hormone	2.0–25.0 IU/L ⁸²
Luteinizing hormone	2.0–75.0 IU/L ⁸²
Progesterone	0.215–52.8 ng/mL ⁷⁷
Estradiol	0.15–14.1 ng/mL ⁷⁷
Testosterone	0.11–17.2 ng/mL ⁷⁷