Critical guidelines for validation of the selectivity of *in-vivo* chemical microsensors

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Microsensors are miniaturized analytical devices for making *in-situ* chemical measurements in biological systems with extraordinary temporal resolution. Their most severe limitation is chemical selectivity. Here, we suggest guidelines that can be applied to a range of *in-vivo* microsensor applications for evaluation of analyte identification and improvement of selectivity.

Keywords: Ascorbate; Biosensor; Dopamine; Glutamate; Voltammetry

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1. Introduction

Our laboratory has developed carbon-fiber microelectrodes that serve as very reliable sensors of dopamine and other neurotransmitters. This electrochemical technology permits chemical measurements in the brain and is revolutionizing the study of chemical signaling by allowing measurements to be made in real time. With such *in-vivo* microsensors, the most severe limitation is their chemical selectivity, and our research has developed ways to circumvent this shortcoming. This concern is common to all sensors used in a biological environment. Indeed, in 1988, a review of *in-vivo* voltammetry listed criteria to aid the chemical identification of signals [1]. These were the consensus of the views of 24 eminent investigators from the international community. However, their use in subsequent work has not been widespread. As a result, there has been some confusion in the literature when published chemical signals have not been adequately characterized. In the meantime, the number of microsensors has broadened and there use has been extended into more complex environments (e.g., the brains of behaving animals). Here, we have reevaluated these original principles in the context of current methodology and experimental preparations to develop guidelines that can be applied to a range of *in-vivo* microsensor applications.

2. What is an *in-vivo* chemical microsensor?

Microsensors are miniaturized analytical devices for making chemical measurements in biological systems. Because analyte separation and detection processes are self-contained, *in-situ* recordings can be made with extremely high temporal resolution (seconds to microseconds [2]). The majority of microsensors employ voltammetry, on which we will focus. However, for the most part, the principles will generalize to methodologies using other detection schemes.

Voltammetric detection involves measuring the current that arises when a molecule changes its redox state. This current is a result of charge transfer during electron gain (reduction) or loss (oxidation). When a potential is applied to an electrode that is sufficient to drive one of these transitions, the current is proportional to the number of molecules electrolyzed (Fig. 1).

Conventionally, voltammetric microsensors have been used to detect small bioactive molecules that are easily oxidized (e.g., monoamine neurotransmitters) or reduced (e.g., oxygen). However, newer technology is emerging for the detection of non-electroactive analytes using more elaborate designs. These are typically

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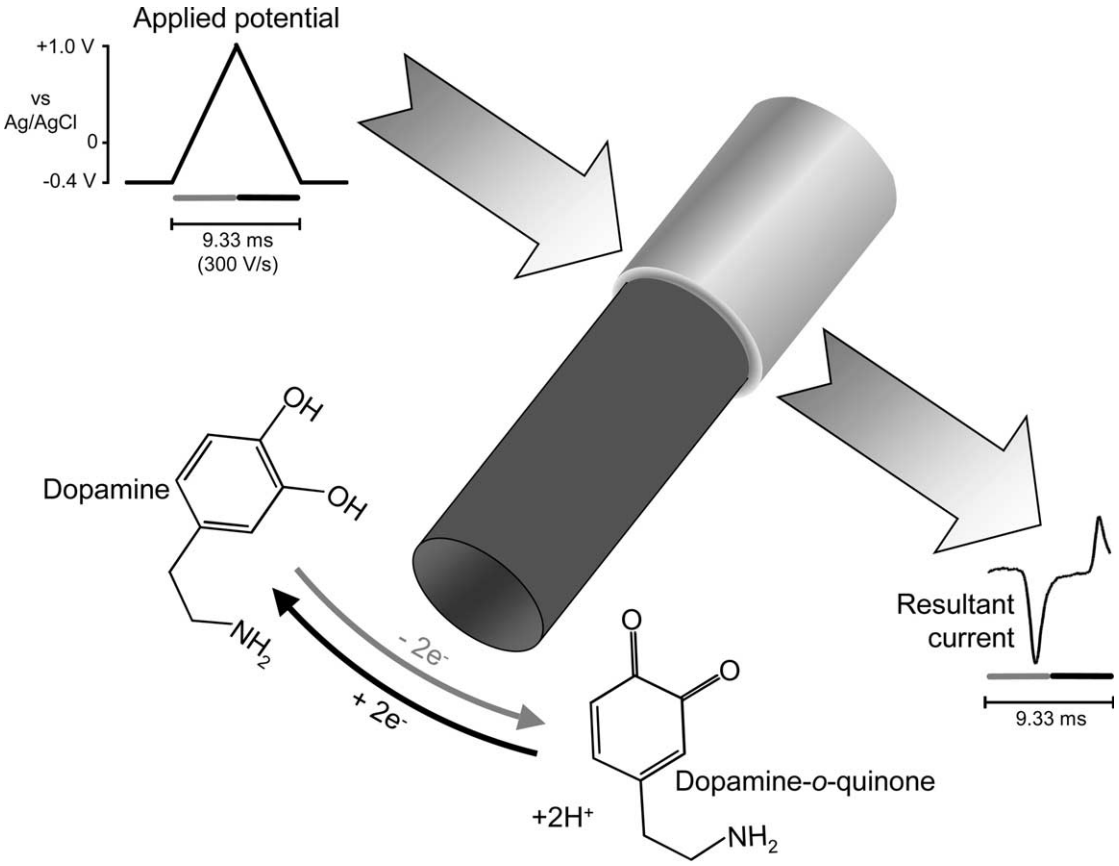

Figure 1. Voltammetric detection of dopamine. When sufficient potential is applied to the electrode, dopamine is oxidized to dopamine-*o*-quinone, donating two electrons that are detected as current. When the potential is returned, any dopamine-*o*-quinone remaining at the electrode surface is reduced back to dopamine by accepting electrons, producing current in the opposite direction. In the example shown, the potential is applied by fast-scan cyclic voltammetry. With this technique, the resultant current comprises time-resolved peaks that aid analyte identification. These measurements are typically repeated several times per second.

biosensors that utilize a biological ''recognition element'' (e.g., an enzyme) in their detection scheme. The recognition element has substrate selectivity for the analyte of interest, and is linked to a co-reaction (''transducer'') which produces an electroactive species that can be detected electrochemically (e.g., Fig. 2).

There are three commonly used potential waveforms that are used to detect electroactive analytes with sub-second time resolution. These are constant-potential amperometry, high-speed chronoamperometry and fast-scan cyclic voltammetry. They are most often used with carbon electrodes, although several biosensors incorporate noble-metal electrodes.

With constant-potential amperometry the electrode is held continuously at a potential that is sufficient to electrolyze the analyte of interest. The current provides a continuous analog record of analyte diffusing to the microsensor. In theory, its temporal resolution is limited only by diffusion and electron-transfer kinetics, although in practice it is also limited by data acquisition and filtering. This technique cannot resolve the chemical identity of the electroactive species.

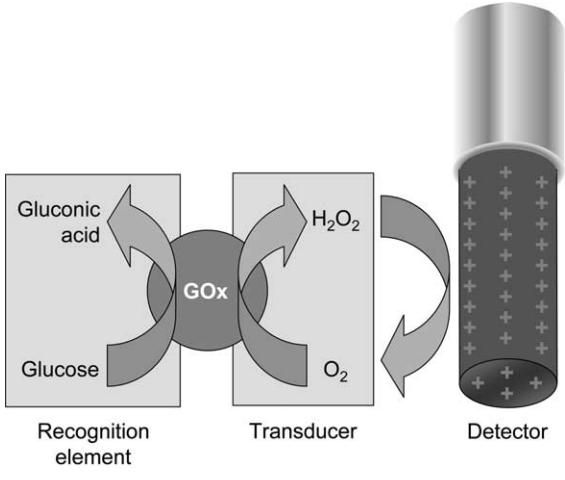

Figure 2. Simplified representation of a biosensor detection scheme for glucose [22]. When glucose is present, it is converted to gluconic acid by glucose oxidase (GOx) that is immobilized on the electrode surface. Secondary to this reaction is the production of the hydrogen peroxide ''transducer'' that is voltammetrically detected when it is oxidized at the electrode.

With high-speed chronoamperometry, the potential is periodically stepped to a level that is sufficient to electrolyze the analyte of interest, held for a finite period and then returned (i.e., a square wave). The ratio of current during the cathodic step (reductive) to that on the anodic step (oxidative) provides a degree of chemical resolution. Although high-speed chronoamperometry can perform at the sub-second time scale, its temporal resolution is ultimately limited by the time required to make discrete measurements.

With fast-scan cyclic voltammetry, the potential is linearly ramped to a level that is sufficient to change the redox state of the analyte of interest and back (i.e., a triangular wave; Fig. 1). During the potential sweep, electroactive compounds produce current peaks that are separated in the time domain (and therefore voltage domain) by differences in their redox properties (formal potential and electron-transfer kinetics) on a millisecond timescale. Thus, fast-scan cyclic voltammetry offers much more chemical resolution. As with high-speed chronoamperometry, its temporal resolution is limited by the requirement to make discrete measurements.

3. Selectivity requirement of a microsensor

The main goals in choosing a microsensor are that it responds to the analyte on the desired timescale and has preferential selectivity for it. Often, a compromise in selectivity is made in favor of temporal resolution (or to simplify data acquisition). The extent to which such a compromise can be justified will depend on the likelihood of interference species being encountered by the microsensor. This will ultimately be governed by the source of the putative analyte as well as the microenvironment around the probe.

3.1. Source of analyte

The confidence in identification of an analyte depends on its source, so this should be considered when choosing a microsensor. For example, when an electroactive species is exogenously applied to the tissue near the microsensor (e.g., iontophoretic application), it is highly likely that the signal originates from this compound, as long as secondary changes (e.g., pH changes) can be excluded. However, to monitor changes in endogenous compounds, much more specificity is needed, since many different compounds are likely changing in concentration. Confidence in analyte identification is greatest when its detection is linked to a stimulus, which should be biologically specific. For example, an agent that causes release *via* binding to a specific receptor (e.g., nicotine) has more selectivity than a general depolarizing agent (e.g., potassium). Likewise, electrical stimulation of a neuronal pathway has more selectivity than local stimulation. The greatest selectivity is required when endogenous compounds are intrinsically evoked (e.g., spontaneous or behaviorally evoked release of a neurotransmitter). Under these conditions, it is particularly important to carry out an extensive characterization of the signal before attributing it to a specific species.

3.2. Recording microenvironment

Critical to the effectiveness of detection of a microsensor is the environment around it. When the microsensor is to be implanted into a complex environment (e.g., in tissue of a whole animal), it is essential that the probe efficiency is relatively insensitive to changes in the environment and can resolve the analyte of interest from these and other chemical changes that may take place. For example, the sensitivity for voltammetrically detected dopamine changes with Ca^{2+} concentration [3], a concern that was eliminated by demonstrating that Ca^{2+} changes were quite small during local electrical stimulation [3,4]. The efficiency of voltammetric dopamine detection is not significantly affected by changes in temperature, pH or oxygen that may occur under physiological or pathological conditions [5,6]. However, pH changes produce a competing signal at the microsensor when using high-speed chronoamperometry [6] or fast-scan cyclic voltammetry [4] (but not constant-potential amperometry; unpublished observations). Therefore, it is important to test the ability of the microsensor to resolve pH from the analyte of interest, particularly when initiating a study where the microenvironment is not under tight experimenter control. For example, we have shown that pH changes are clearly discernible from dopamine using fast-scan cyclic voltammetry [7].

Temperature fluctuations are a particular problem for biosensors because the biological recognition element component is almost certainly temperature dependent. However, this issue can be minimized by restricting diffusion with a polymer [8]. This delays the delivery of the analyte so that the rate-limiting step is diffusion rather than the biological recognition element. Under these conditions, the effect of physiological temperature changes on the efficiency of detection should be less.

4. Evaluating and improving chemical selectivity

With voltammetric detection, chemical selectivity can be achieved by two means- selective sensitivity or chemical resolution. For example, both constant-potential amperometry and fast-scan cyclic voltammetry can selectively measure dopamine changes during fluctuations in pH, but for different reasons.

Constant-potential amperometry is relatively insensitive to pH compared to dopamine, so it measures only the change in dopamine (selective sensitivity). Conversely, fast-scan cyclic voltammetry detects changes in pH, but these are electrochemically separated from dopamine (chemical resolution). With all microsensors, a complete characterization should be made of the responses to the analyte of interest and a series of compounds that may interfere *in vivo*.

Biosensors often have exquisite selectivity for the primary analyte because of the specificity of the biological recognition element. However, the secondary process of detection of the transducer (e.g., H_2O_2) may not be so selective, so it is during electrochemical detection that interference is most likely.

4.1. Selective chemical sensitivity

Because current results from every type of molecule that is electrolyzed, voltammetric detection is inherently non-selective. However, several strategies have been used to overcome this. Selectivity can be enhanced by coating electrodes with ion-selective polymers, such as Nafion [9], which improves the selectivity for cation analytes, since it allows the passage of cations to the electrode while excluding anions and larger molecules. Similar selective enhancement of sensitivity can be achieved through adsorption [10]. This can be promoted by electrochemical oxidation of the carbon surface [11]. Selectivity has also been improved by incorporating onto the electrode an enzyme that can eliminate electroactive interference species [12].

There are a number of lesser-used voltammetric techniques that utilize differential measurement of current at two potentials at the *same* electrode. For example, differential pulse amperometry uses a pre-pulse potential that is not sufficient to electrolyze the analyte, followed by another that is. Subtraction of the current obtained at the first potential from that at the second should, in principle, remove the interference from species electrolyzed by the pre-pulse. An equivalent differential measurement can be made using fast-scan cyclic voltammetry. Since the potential is linearly scanned between two limits, the current at any potential in this range can be subtracted from that at any other. For maximum versatility, this can be accomplished off-line with a computer. We have found this particularly useful for removing pH interference from dopamine measurements, thus increasing the selectivity for dopamine [7].

An important advance in the design of biosensors is the use of a differential measurement *between* two electrodes [13]. The ''self-referencing'' technique uses one biosensor with and one without a biological recognition element, thus allowing the removal of all non-specific current. For this strategy to work, the two electrodes must be electrically and physically equivalent (i.e., have the same bare electrode size and film thickness of any polymer layer). This goal can be achieved with the use of microfabrication techniques [14]. Since there is a finite spatial separation between the two electrodes, it best suited to microenvironments when the chemistry is spatially homogeneous at that level.

4.2. Chemical resolution

Microsensors that respond to multiple analytes must be capable of resolving them. With fast-scan cyclic voltammetry, chemical resolution can be assessed using the cyclic voltammogram. This current-voltage curve is the ''chemical signature'' used for identification. Since the position of the current peaks is a function of the formal potential, electron-transfer kinetics and the reversibility of the redox reaction, there can be separation of competing analytes.

Traditionally, the cyclic voltammograms for different species have been compared, and their discrimination has relied on the investigator's judgment. However, to ensure objectivity, we compare cyclic voltammograms of the analyte of interest and that of other compounds with linear regression and obtain a correlation coefficient. This provides a useful cut-off, above which one can have confidence that the signal is not that specific contaminant. For example, if the dopamine metabolite, DOPAC, achieves a correlation coefficient of $r^2=0.5$ *versus* dopamine *in vitro*, it is unlikely that an *in-vivo* signal that attains $r^2=0.9$ *versus* dopamine could be attributable to DOPAC. Similar strategies can be employed with the reduction–oxidation current ratio obtained during high-speed chronoamperometry. However, since there is only a single ratio, as opposed to a series of peaks with cyclic voltammetry, high-speed chronoamperometry is inherently less selective.

Constant-potential amperometry has no chemical resolution among species where the redox states are changed by the applied potential. However, one approach has been to use fast-scan cyclic voltammetry first to identify a signal, and then to switch to constant-potential amperometry (at the same electrode) [15]. This has the advantage of combining the superior chemical resolution of fast-scan cyclic voltammetry with the microsecond time resolution of constant-potential amperometry. Because electrochemical analyte identification can be accomplished only before (and possibly after) the experiment, this approach can be used only for signals that have been shown to be reproducible throughout an experimental session (e.g., iontophoretic analyte application, electrically-evoked neurotransmitter release, or vesicular release from a single cell). However, with behaviorally evoked signals, reproducibility cannot be guaranteed from trial to trial because habituation, learning, changes in the environment and perhaps even cognition can have an effect,

and thus, this approach is not suitable for these types of experiments.

5. Characterization of *in-vivo* signals

Once a suitable microsensor has been chosen and its responses characterized, it is necessary to verify that *in-vivo* signals do indeed reflect extracellular changes in the purported analyte. Electrochemical verification of the signal is the most direct means of signal identification, and therefore perhaps the most important. Nonetheless, because this cannot provide unequivocal selectivity, it is essential to corroborate the electrochemical information by indirect methods using multiple criteria.

These criteria are intended to be applied to the *in-vivo* signal of interest *per se* rather than a general characterization of the electrode (which should also be carried out). Just because a microsensor can respond to a particular species, does not mean that all signals it detects can be attributed to that species. If the signal of interest is a chemical change in the brain during a lever-press response for heroin, it is the signal during that behavior that must be characterized. Likewise, every new signal requires a new characterization. This can be tedious, especially for behaviorally-evoked signals where many behaviors may be studied, but is absolutely imperative if the data are to be believable. For example, if the signal when the animal presses the lever for heroin can be characterized as dopamine, this does not negate the requirement to characterize a signal for a lever press for cocaine or food, or even a nose poke for heroin.

5.1. Electrochemical verification

Current can arise from multiple sources, including electrolysis, double layer charging, movement artifacts, and changes in tissue impedance. It is therefore critical to demonstrate that the voltammetric signal is consistent with the analyte of interest. The extent to which analytes can be identified differs between the detection techniques (fast-scan cyclic voltammetry > high-speed chronoamperometry > constant-potential amperometry), therefore the level of confidence one places in the assignment should reflect this.

With fast-scan cyclic voltammetry, the cyclic voltammogram of an *in-vivo* signal should attain a high correlation coefficient when compared to an *in-vitro* standard of the purported analyte. In addition, it can be useful to compare the cyclic voltammogram to another *in-vivo* signal that is well-characterized as the analyte of interest. This has the advantage that it allows the signal to be evaluated against a standard that was obtained at the same recording site (i.e., with the same electrolyte, tissue impedance and noise). We used this strategy to provide evidence for sub-second changes of dopamine in the nucleus accumbens during cocaine-self-administration behavior by demonstrating that the electrochemical signals had correlation coefficients of greater than r^2=0.9 compared to signals obtained by electrically stimulating dopaminergic neurons [16]. Likewise, with high-speed chronoamperometry, the reduction-oxidation current ratio of an *in-vivo* signal should be similar to an *in- vitro* standard of the purported analyte and/or a well-characterized *in-vivo* signal.

With constant-potential amperometry, it should be demonstrated that the *in-vivo* signal can be abolished when the electrode is held at a potential that is insufficient to electrolyze the purported analyte. Ideally, signals will be electrochemically verified using fast-scan cyclic voltammetry before and/or after the experiment (see above). When using biosensors, the selectivity should be tested by carrying out a control experiment using an equivalent microsensor without the biological recognition element present [17]. This could be a second electrode on a multisite biosensor [13]. If there is still signal present in the absence of the enzyme, and this cannot be chemically resolved from the transducer, the use of a multisite biosensor in differential (self-referencing) mode [13] should be considered. However, when using a self-referencing biosensor, it should be confirmed that there is indeed no signal when both electrodes are devoid of enzyme.

5.2. Anatomical and physiological verification

Sufficient quantities of the purported analyte should be present in the tissue where the recording is made. This can easily be confirmed by tissue-content measurements. In the case of a neurotransmitter, the recording area should be innervated by cells that can release that transmitter. This can be verified with post-mortem histological methods for whole-animal experiments, visually for slice experiments where the anatomy is established or during tissue harvesting where explanted tissue will be used.

In addition, the purported analyte should be capable of changing concentration at the rates measured. This can be estimated from the tissue content and the known kinetics of the physiological processes involved. Where spontaneous or behaviorally-evoked chemical changes are being measured, the conceivable rate of concentration change can be verified by exogenously evoking a change. For example, as proof of principle, we have confirmed by electrically evoking dopamine release that dopamine can rapidly change its concentration at the recording sites where we observe rapid changes the electrochemical signal during behavior [16,18,19]. Furthermore, the case will be strengthened if there is additional corroborative evidence for the purported chemical change to take

place, such as electrophysiological data under equivalent experimental conditions.

5.3. Pharmacological verification

The signal should respond to known pharmacology in a predictable manner. Drugs particularly useful for such investigations include inhibitors of synthesis, metabolism, and storage. Enzymes have also been demonstrated to be a useful. For example, in the detection of ascorbate, ascorbate acid oxidase was used to eliminate the signal [20].

When studying a behaviorally-evoked signal, global alteration of that signal by systemic administration of drugs would almost certainly change the very behavior itself. Under these circumstances, drugs should be applied locally, close to the recording electrode (e.g., by iontophoresis). This should allow the desired alteration of neurotransmission at the spatial level of the recording without a significant disruption of behavior.

5.4. Independent chemical verification

To confirm the identification provided by the sensor, an independent chemical analysis is important. This is especially the case for microsensors or techniques with little chemical selectivity. An example described above is the use of fast-scan cyclic voltammetry with constant-potential amperometric recordings made at the same site. Another example is the use of a selective adsorbent probe based on alumina to extract dopamine from brain tissue adjacent to an electrode [21]. Such chemical verification may be difficult to achieve when the chemical microsensor responds on rapid time scales that are inaccessible to other techniques. For example, a microdialysis sample is typically obtained over 5 minutes. This type of sampling will average out dopamine fluctuations seen on a sub-second time scale with voltammetry.

6. Conclusions

In order to make reliable measurements with *in-vivo* chemical microsensors, a number of steps are necessary. Great care should be placed in choosing a microsensor that not only can perform on the desired timescale, but also has sufficient selectivity for the analyte of interest for the specific experimental conditions. This selectivity should be confirmed by *in-vitro* characterization of the microsensor with the analyte of interest and other species that could interfere *in vivo*.

When a microsensor is used experimentally, it should be used in a region where there is circumstantial (anatomical and physiological) evidence for the presence of the analyte of interest, and the electrochemical signal should be consistent with this species. There should also be independent corroborative evidence for the signals seen, gained with alternative technology. Finally, the signal should be characterized by appropriate pharmacological intervention.

Once all of these steps are taken to avoid erroneous identification of analytes, the benefits of *in-vivo* chemical microsensors can be enjoyed to their full.

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References

[1] C.A. Marsden, M.H. Joseph, Z.L. Kruk, N.T. Maidment, R.D. O'Neill, J.O. Schenk, J.A. Stamford, Neurosci. 25 (1988) 389.
[2] E.N. Pothos, V. Davila, D. Sulzer, J. Neurosci. 18 (1998) 4106.
[3] J. Kume-Kick, M.E. Rice, J. Neurosci. Methods 84 (1998) 55.
[4] S.R. Jones, G.E. Mickelson, L.B. Collins, K.T. Kawagoe, R.M. Wightman, J. Neurosci. Methods 52 (1994) 1.
[5] K.T. Kawagoe, P.A. Garris, R.M. Wightman, J. Electroanal. Chem. 359 (1993) 193.
[6] G.A. Gerhardt, A.F. Hoffman, J. Neurosci. Methods 109 (2001) 13.
[7] B.J. Venton, D.J. Michael, R.M. Wightman, J. Neurochem. 84 (2003) 373.
[8] D.S. Bindra, Y. Zhang, G.S. Wilson, R. Sternberg, D.R. Thevenot, D. Moatti, G. Reach, Anal. Chem. 63 (1991) 1692.
[9] G.A. Gerhardt, A.F. Oke, G. Nagy, B. Moghaddam, R.N. Adams, Brain Res. 290 (1984) 390.
[10] B.D. Bath, D.J. Michael, B.J. Trafton, J.D. Joseph, P.L. Runnels, R.M. Wightman, Anal. Chem. 72 (2000) 5994.
[11] F. Gonon, M. Buda, R. Cespuglio, M. Jouvet, J.F. Pujol, Nature (London) 286 (1980) 902.
[12] G. Nagy, M.E. Rice, R.N. Adams, Life Sci. 31 (1982) 2611.
[13] J.J. Burmeister, G.A. Gerhardt, Anal. Chem. 73 (2001) 1037.
[14] J.J. Burmeister, F. Pomerleau, M. Palmer, B.K. Day, P. Huettl, G.A. Gerhardt, J. Neurosci. Methods 119 (2002) 163.
[15] R.M. Wightman, J.A. Jankowski, R.T. Kennedy, K.T. Kawagoe, T.J. Schroeder, D.J. Leszczyszyn, J.A. Near, E.J. Diliberto Jr., O.H. Viveros, Proc. Natl. Acad. Sci. USA 88 (1991) 10754.
[16] P.E.M. Phillips, G.D. Stuber, M.L.A.V. Heien, R.M. Wightman, R.M. Carelli, Nature (London) 422 (2003) 614.
[17] N.V. Kulagina, L. Shankar, A.C. Michael, Anal. Chem. 71 (1999) 5093.
[18] D.L. Robinson, P.E.M. Phillips, E.A. Budygin, B.J. Trafton, P.A. Garris, R.M. Wightman, Neurorep. 12 (2001) 2549.
[19] D.L. Robinson, M.L.A.V. Heien, R.M. Wightman, J. Neurosci. 22 (2002) 10477.
[20] J.O. Schenk, E. Miller, R.N. Adams, Anal. Chem. 54 (1982) 1452–1454.
[21] M.E. Rice, A.F. Oke, C.W. Bradberry, R.N. Adams, Brain Res. 340 (1985) 151.
[22] M.G. Boutelle, C. Stanford, M. Fillenz, W.J. Albery, P.N. Bartlett, Neurosci. Lett. 72 (1986) 283.