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Review Article

Nanoelectrochemical analysis inside a single living cell

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Abstract

This critical opinion reviews the methods to construct and apply electrodes for analysis inside single cells and of single organelles. Nanoelectrochemical methodology, with an emphasis on nanoelectrode construction and analysis of metabolites, neurotransmitters, reactive oxygen and nitrogen species, glucose, oxygen, hydrogen peroxide, and ions in cells are discussed for measurements from cytoplasm to single organelles.

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Nano-electrodes, Intracellular, Vesicle, Phagolysosome, Neurotransmitter, Reactive oxygen species.

Introduction

Electrochemical techniques have been applied for use in biomedical research. As the essential element of life, the single cell is a consistent focus of biomedical investigations and is also an important target for electrochemical analysis. Considering that most physiological processes occur within cells, measurements inside single cells obviously provide more 'high fidelity' information reflecting the key molecules real-time level in situ and their metabolism affected by complete cell environment. However, because of the small size of most cells and fragile viability, extremely small measurement probe sizes are required. Additionally, these need to be capable of measuring low quantities (sub μM) of analytes and fast (sub second) cellular processes. Nanoelectrochemical analytical techniques are powerful tools for intracellular detection because of their distinct advantages: powerful quantitative ability, low to nM detection limits, fast to sub-ms response time, and maintaining cell viability during cell implantation. This review will summarize the development and key applications of several nanoelectrochemical analytical techniques for intracellular analysis.

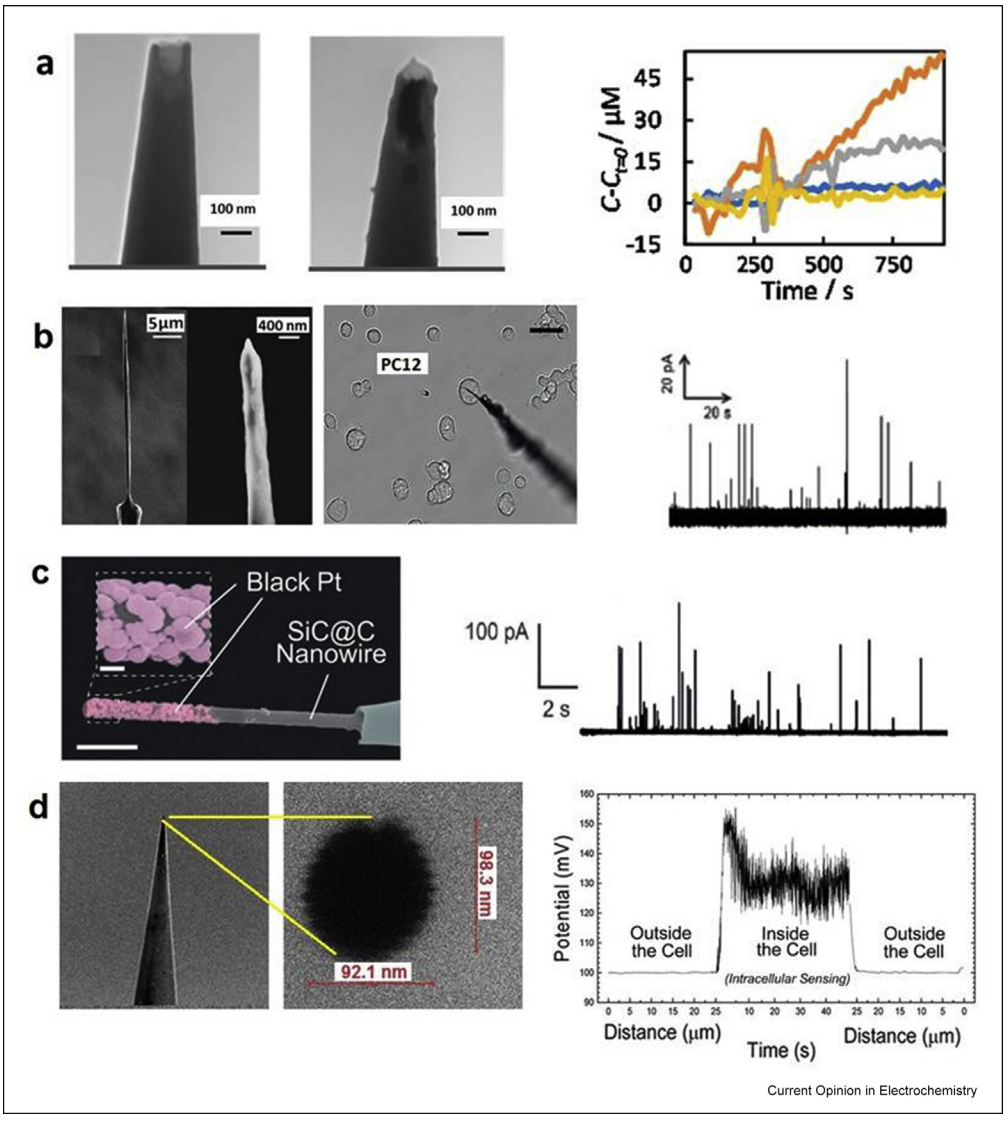
Nanoelectrochemical methodology

In the majority of reports of intracellular electrochemical analysis, by using a suitable manipulator, the tip of the small electrode is first placed gently on top of the cell membrane while observing in an optical microscope. Then, the electrode is slowly pressed through the cell membrane while the potential or current is recorded. The aim is to insert the electrode into the cell without severely damaging it, and hence, it is essential to reduce the electrode size down to the submicrometer regime, although historically some slight larger micrometer electrodes were applied in giant cells. A reproducible and time-saving protocol to fabricate these tiny, best if durable, electrodes is the key to all successful probe construction methods. Some examples are shown in Figure 1 and summarized in Table 1.

Basic nanoelectrode fabrication

Early ideas to construct nanoscale electrodes for intracellular detection aimed at filling or coating a prepulled glass micropipette. In 1967, an intracellular sensor with 4 μm diameter was used to realize the detection of oxygen (O_2) [1] in gracilis muscle cells. This sensor was made by filling a glass micropipette with an alloy of Wood's metal and gold. In 1986, Kim et al. deposited carbon layers inside a micropipette to fabricate carbon microring electrodes by pyrolysis of hydrocarbon gases as a carbon precursor [2]. And, in the same year, Meulemans et al. carried out intracellular voltammetry with a microcarbon electrode which was fabricated by sealing an electrochemically etched carbon fiber in a micropipette and exposing a disk as the electrode surface [3]. Recently, Mirkin et al. made some even smaller nanoelectrodes (Figure 1a), having 40-nm diameter [4,5]. These electrodes were constructed by making a nanocavity of pyrolytic carbon by chemical vapor deposition in a pipette tip and filling it with Pt by electrochemical deposition [4–7]. Noble metal sputtering was also used by Pan et al. to form a Pt layer on the outer and inner walls of a nanopipette tip to construct a nanoelectrode

Figure 1



Microscopic images of example nanoelectrodes, cellular sites, and obtained typical responses (a) Carbon-filled pipet before (left) and after platinum deposition and differences of the responses to ROS/RNS (H_2O_2 [red curve] ONOO [purple curve], NO• [green curve], and NO_2 [blue curve]) inside the target cell at different potentials [4] (b) Nano-tip CFEs (scale bar: 400 nm) and magnified view of the tip of nano-tip CFE (scale bar: 400 nm) and amperometric traces of vesicular content in a cell [13] (c) SiC@C@Pt nanoelectrode with measurement of reactive oxygen species in individual phagolysosomes [18] (d) Modified nanopipette with GOX and its intracellular measurement [23]. Reproduced with permission from the American Chemical Society, Wiley- VCH Verlag GmbH & Co., Elsevier and the Royal Society of Chemistry.

(see section 1.3) [8], and Ying et al. used a similar approach but formed a Au layer only on the inner wall of the nanopipette tip [9]. In general, the success of this method depends on the accurate control of the pipette size, deposition stability, insulation, and electrical connection [10].

In addition to development of techniques where electrodes are fabricated from the 'bottom-up', an alternative strategy is to reduce the electrode size. An excellent example is flame etching which has been used to produce nanoscale carbon fiber electrodes (CFEs) [11]. Flame etching to make small electrodes was pioneered by the Li et al. to make conical 'nano-tip' CFEs [12], which have a high aspect ratio able to maintain the relatively bigger electrode surface while the cell wound during insertion remains small. This protocol is a simple and effective way to make CFEs for intracellular analytical techniques. Intracellular vesicle impact electrochemical cytometry with flame etched CFEs

Table 1

Nanoelectrodes for intracellular analysis.

Electrode	Target	Cell type	Dimension	Geometry	Detection method	Ref.
Wood's metal and gold in micropipette	Oxygen	Gracilis muscle cells of guinea pig	D: 1–2 μm	Disk	Amperometry	[1]
Flame etched carbon fiber electrode 'Nano-tip'	Catecholamines	PC12	D: 500 nm tip diameter: ~50 nm	Conical	Amperometry	[13–15,44,49]
Electrochemically etched carbon fiber electrode	Catecholamines	PC12	L: 5 μm; tip diameter: ~300 nm	Conical	Amperometry and Fast-scan cyclic voltammetry	[16]
Platinized carbon nanoelectrodes	ROS and RNS	Noncancerous and metastatic human breast cells	D: 80 nm	Disk	Voltammetry and Amperometry	[4]
		Macrophages	D:≤100 nm)			[6,7]
Modified nanopipettes	ROS and RNS	Macrophages	D:≤400 nm)	Nanopipette	Amperometry	[33]
Carbon nanoelectrodes/ Prussian Blue	Hydrogen peroxide	Murine macrophage J774A.1 Human embryonic kidney tsa201 cells	D: 50–200 nm	Disk	Amperometry	[22]
Modified nanopipettes	Glucose	MDA-MB-231 and MCF-7[a]	D: ~90 nm	Nanopipette	Potentiometry	[23]
	pH	Human fibroblasts, HeLa, MDA-MB-231 and MCF-7	D: ~100 nm			[24]
Platinized SiC@C nanowire	ROS and RNS	Macrophages	L:10 μm D: 300–600 nm	Cylindrical	Amperometry	[17,18]
	Mitochondrial ROS	NIH 3T3 cell	L:10 μm D: 500–600 nm	Cylindrical	Amperometry	[36]
Enzymatic platinized SiC@C@Au nanowire	Glucose	Human umbilical vein endothelial cells (HUVEC)	L:10 μm D: 500–600 nm	Cylindrical	Amperometry	[21]
Glass microcapillary/ nanoflake ZnO	Glucose	Human adipocytes and frog oocytes	Not known	Cylindrical	Potentiometry	[52]
Glass microcapillary/ ZnO nanowire/K^+ membrane	K^+	Human oocytes	D: 100–180 nm	Disk	Potentiometry	[50]
Composite electrode[b]	Hydrogen peroxide	Hela cells	D: 1–2 μm	Disk	Electrochemilu-minescence	[27]
Platinum ('Nano-kit')	Hydrogen peroxide	Protein activity or glucosidasee activity	Pt layer thickness: 70 nm D:200 nm	Hollow ring	Amperometry	[26]
	Glucose	HeLa cells				[8]
	Phosphate ion	HeLa cells				[25]

PC12, Pheochromocytoma cells; ROS and RNS, reactive oxygen/nitrogen species.

[a] Breast cancer cell lines.

[b] Mixture of chitosan and luminol/polyvinyl chloride/nitrophenyloctyl ether and gold layerantipyrine.

(Figure 1b) is an approach to quantify catecholamines in individual vesicles inside living cells [13–15]. Owing to the importance role of the vesicles in cellular communication, analysis of neurotransmitters stored inside vesicles is highly important. Another more recent approach to carry out vesicle measurements with insulated nanotip electrodes and fast scan voltammetry has been reported [16]. This approach while providing an edge in identification of species measured is not able to quantify the number of molecules in each vesicle.

The direct integration of a single nanowire into a nanosensor is a novel strategy for making nanoelectrodes but is to date rarely reported for applications in single cells. Zhang et al. placed a single carbon coated silicon carbide 'core–shell' nanowire (diameter: 300–500 nm) in the orifice of a nanopipette to construct a single nanowire electrode (Figure 1c) which was coated with Pt nanoparticles as nanocatalysts to improve the sensitivity for intracellular reactive oxygen species/ reactive nitrogen species (ROS/RNS) measurements [17,18]. These works provided an alternative strategy for making high aspect ratio nanoelectrodes and opened a new window for employing new nanomaterials to the limited material library of nanoelectrodes for cellular measurements.

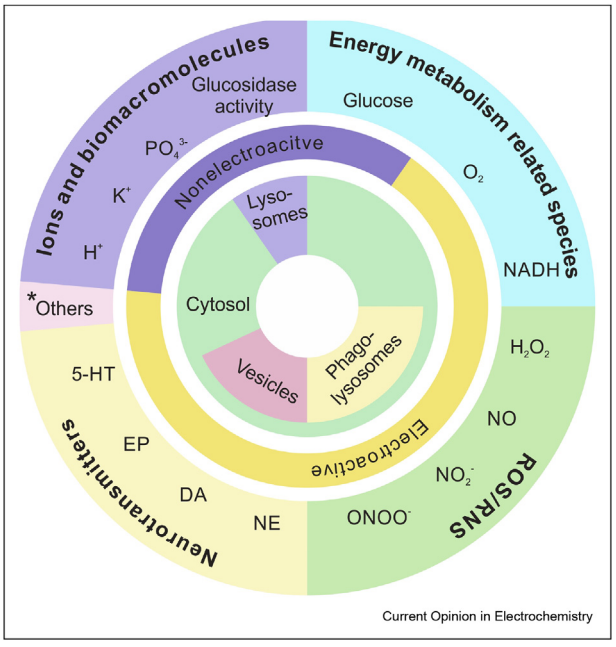
Modification of electrodes

The modification of an electrode surface with an enzyme or nanomaterial is a widely used approach to enhance the selectivity, sensitivity, and extend the analytical scope of electrochemical measurements [19], especially for those targets with low electroactivity. Early work, before the nanorevolution, reported the development enzymatic microcarbon-ring electrodes to monitor the glucose inside cells [20], and more recently, Liao et al. did similar experiments with smaller single nanowire electrodes [21]. Marquitan et al. electrochemically deposited Prussian blue on carbon nanoelectrodes as an electrocatalyst, which enabled selective intracellular H_2O_2 detection without the interferences caused by ascorbic acid and molecular oxygen [22]. Nascimento et al. developed a nanopipette modified with glucose oxidase which varies the local pH and electrode potential (Figure 1d) in the presence of intracellular glucose [23]. Similarly, chitosan as a pH-sensitive polymer was placed inside hydroxylated quartz nanopipettes for sensing intracellular changes in pH [24]. However, the introduction of new material to such miniscule electrodes while maintaining the homogeneity and dimension of the sensors is still highly challenging.

Combination of methods

In addition to the common modification procedures discussed above, designing combined electrodes increases the broad range of applications of nanosensors in intracellular sensing. A tool called a 'nano-kit' for single cell analysis has been developed by Pan et al. [8]. The kit is made by depositing a Pt layer in the inner wall of a hollow nanopipette as an electrode surface and filling the nanopipette with a commercial assay kit (femtoliter) for glucose detection. After inserting the electrode into a cell, the assay kit solution can be pushed into the cytosol where it reacts, generating H_2O_2 via an enzymatic reaction which is then converted into an electrochemical signal. They used this nano-kit to detect phosphate [25] and glucosidase activity [26] by changing the components of the filling solution. They also combined electrochemiluminescence at a microelectrode to carry out intracellular sensing. In this case, the sensor consisted of a capillary (tip opening: 1–2 μm) filled with a mixture of chitosan and luminol, which was coated with a gold-coated porous polymer layer. Electrochemiluminescence signals were generated when cytosolic H_2O_2 interacted with the luminol–chitosan mixture [27].

Figure 2



Schematic overview of the target analytes for intracellular electrochemical detection. The outer circle: the detected species; middle circle: the electroactivity of target analytes, the modification of electrodes, or combination of electrochemical sensing and other analytical methods, is necessary to detect nonelectroactive species; inner circle: the locations of target analytes within cells. *Others [3]: ascorbic acid, metronidazole, antipyrine.

Target analytes inside a living cell

In this section of the review, we provide an overview of the target analytes measured inside living cells. Usually, these targets are widely distributed in an organism and involved in several physiological processes, such as

energy metabolism, intercellular communication, immune response, and oxidative stress. When target molecules are electroactive, as mentioned above, rapid regulation of these targets can be monitored by nanoelectrochemical techniques with advantages of high temporal resolution and quantitative ability.

A graphical overview of target analytes for intracellular analysis is depicted in Figure 2. We classify the common target species for intracellular electrochemical detection based on their related research topics, electroactivity, and distribution inside the cells. Major analytes are electroactive so that they are easily detected by microelectrodes/nanoelectrodes, whereas some modification of the electrode is necessary to measure nonelectroactive analytes such as glucose, inorganic ions, and enzymes. Except for glucosidase, almost all analytes exist in the cytosol, whereas some are resourced or concentrated more in specific organelles to carry out their functions, like ROS/RNS in phagolysosomes and some neurotransmitters in vesicles.

Cellular analysis of species related to energy metabolism

Classic cellular target molecules to understand energy metabolism include O_2, glucose, NADH, etc. These are all involved in the cell respiratory chain and are critical to cell function. In 1990, Uchida et al. used a noble metal ultramicro-ring electrode to monitor the intracellular oxygen concentration inside a living protoplast of *Bryopsis plumosa* [28]. The Lau et al. recorded the oxygen level fluctuation in giant dopamine neuron of *Planorbis corneus* and demonstrated its dynamic linkage to the surrounding oxygen level [29]. In the same year, they also used small electrochemical sensors to estimate the cytosolic glucose concentration of a neuron the high micromolar range [20]. With an increasing interest in diabetes and cancer, Asif et al. used functionalized ZnO nanorod–based electrodes to report the cytosolic glucose level in oocytes and that it doubled after stimulation with insulin [30]. Pan et al. utilized the 'nano-kit' described above to suggest the intracellular glucose level decreases in starved HeLa cells [8]. Nascimento et al. used a glucose nanosensor to show that the glucose level (~mM) in cancer cells is 2–5 times higher than in nonmalignant cells (~submM) [23], supporting the hypothesis that cancer cells have higher metabolic activity and a greater need for glucose. Liao et al. used nanowire sensors to show that intracellular glucose levels in human endothelial cells increase 1.5-fold after glucose incubation, providing direct evidence that human fibroblast growth factor 1, used as a hypoglycemic drug, facilitates uptake of glucose in hepatocytes [21]. Finally, intracellular NADH regulation was also quantified by use of nanopore electrodes for imaging cells, and NADH generation was shown to be inhibited by Taxol, an anticancer drug, in cancerous cells [9].

Cellular measurements of reactive oxygen/nitrogen species

Small molecules including $O_2^{\cdot-}$, H_2O_2, NO, *etc.* comprise the so-called ROS/RNS species and are involved in cell–cell signal transmission, aging, immune reactions, chronic disease initiation, and cellular defense mechanisms. ROS species have been measured at single cells since the 1990s [31,32]. With the advent of better nanoelectrodes in the last decade, several advances have been made. Wang et al. adopted a platinized nanodisk electrode to monitor the leakage of ROS/RNS from phagolysosomes and rapid clearance in phagocytic cytoplasm [7]. Further, they identified multiple ROS/RNS species inside living cells and recorded their real-time production [4], even making measurements at the single phagolysosome level [6,33]. Combined with measurements of ROS/RNS homeostasis in individual phagolysosomes monitored by platinized single nanowire nanoelectrodes [17,18], a model of the dynamic generation, homeostasis, and regulation of intracellular phagolysosomal ROS/RNS species is emerging. This model benefits the understanding of the congenital inflammatory response and its induction of chronic disease. Although RNS are not generated in mitochondria, ROS are and these have been studied in cells other than phagocytes. Xu et al. used nanopipette electrodes to report a uniform distribution of cytosolic ROS in the neuronal cell body and axons [34]. Subsequently, they used this approach to observe the dynamics and spatial distribution of mitochondrial ROS within noncancerous and cancerous cells after they were treated with inflammation factor and capsaicin [35]. Jiang et al. also used nanowire electrodes to study mitochondrial ROS generation induced by anticancer drug, paclitaxel, and suggested that paclitaxel targets the complex IV of the respiratory chain and the higher paclitaxel-susceptibility of cancer cells may result in the selectivity of paclitaxel [36].

Measurements of neurotransmitters in cells

As a class of essential messengers during cellular communication, neurotransmitters, such as serotonin, dopamine, *etc*, are important targets for electrochemical analysis. More recently, with the advent of smaller electrodes, it has become possible to electrochemically measure storage and regulation inside cells.

As early as 1987, Meulemans et al. began to apply voltammetry to monitor intracellular serotonin levels as they increase with cellular intake of its precursor, L-tryptophan in a living ganglia cell from *Aplysia california*. These experiments started a new direction in cellular electrochemistry, although the neurons used were the giant cells with diameters of 200–300 μm [37]. Chien et al. used intracellular voltammetry to show that dopamine is highly enriched (>98%) inside vesicles, while low in the cytosol [38]. Lindau et al. developed 'patch amperometry' to study the intracellular homeostasis of catecholamines and its rapid regulation through a series of enzymes [39,40].

Recently, Li et al. showed that intracellular vesicle impact electrochemical cytometry could be used to quantify the catecholamines within individual vesicles [13], and this has been applied already. Compared with the exocytotic quantities of catecholamine, the higher intravesicular catecholamine quantities strongly support the 'partial release' hypothesis [41–43]. And, by comparing release to vesicle content, follow-up work has provided more details about the role of Zn^{2+} [44,45], ATP [46], repetitive stimuli [47], anticancer [48], and addictive drugs [14,15,48] in regulating the partial opening dynamics during exocytosis.

Cellular levels of ions and biomacromolecules

Owing to poor electroactivity of inorganic ions and biomacromolecules, the electrochemical measurement of these species in cells is relatively rare. Willander et al. reported that ZnO nanowire potentiometric sensors can be used to estimate the pH and K^+ in fat cells and oocytes, respectively [50,51]. On the basis of the rectification signal in nanopore electrodes, Ozel et al. compared the intracellular pH between noncancerous and cancerous cells. The results directly showed that the pH is lower in cancer cells resulting from a higher metabolic activity promoting production of acidic species and CO_2 [24]. Moreover, Xu et al. applied the 'nano-kit' to estimate the intracellular PO_4^{3-} concentration to be in the mM range [25]. Through a similar strategy but by sorting single lysosomes *in situ*, this group also determined the lysosomal glucosidase activity and their homogeneity within single living cells, a novel finding [26].

Conclusions and perspective

Nanoelectrochemistry provides a powerful repertoire for quantitative information about (bio)chemicals inside individual living cells, and even in single organelles, with high temporal resolution. As electrode size decreases and the aspect ratio increases, these electrochemical sensors have attracted more attention to applications of intracellular analysis. The main areas have been quantification of various biomolecules and ions related to energy metabolism, oxidative stress, neurotransmitter regulation, *et cetera*.

Even though the idea of 'intracellular electrochemical analysis' has been proposed for half of a century, the difficulty in making the small probes and measuring small currents still limits its application. Open questions about these measurement approaches that still need be addressed include electrode fouling and a weak ability to identify the structure of species measured. However, another direction that could bring some excitement would be the simultaneous measurement of individual organelle size and content. Our group as well as the Mirkin group at CUNY and Zhang group at Washington are working on this with some limited early successes, but not fully successful inside cells to date. It will also be very exciting to be able to measure and identify several species at once in cellular organelle, and these experiments appear to be on the horizon. More difficult tasks will be to dissect the chemistry spatially across the inside or surface of a single vesicle. The early combination of nanoelectrode experiments with NanoSIMS promise to help here, but a lot is left to be done [53,54]. Finally, there is continually an issue with quantifying nonelectroactive substances in subcellular and organelle environments. The issues here is to gain a selective response that is sensitive and fast enough to work in this environment. There are a few preliminary reports of sensors fast enough, but not intracellularly, and the work here is varied and at times a bit suspicious. Time will tell. The future is bright in this area mainly because the remaining questions are many and highly important.

Declaration of Competing Interest

Nothing declared.

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