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Modern Electrochemistry in Nanobiology and Sensorics

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1. Introduction

1.1. Overview

Nanotechnology is an area which has promising perspectives for directing fundamental research into successful innovations. Not only to increase the competitiveness of industry but also to create new products that will make positive changes in the lives of citizens, be it in medicine, environment, electronics or any other field [1-4]. Nanoscience and nanotechnology open up new avenues of research and lead to new, useful, and sometimes unexpected applications. Novel materials and new-engineered surfaces allow making products that perform better [5]. New medical treatments are emerging for many diseases [6]. Computers are built with nanoscale components [7] and improving their performance depends upon shrinking these dimensions yet further.

The requirements of nanoscience and nanotechnology have led to modern developments in i.e. electrochemistry. The biological phenomena such as the cellular redox environment, release of neurotransmitters and other signaling substances based on exocytosis, and cellular adhesion are vital elements in modern nanotechnology [8]. Next important problems are the capabilities of electrochemical amperometric and impedance spectroscopic techniques in monitoring cellular dynamics. The applications of such techniques already include biosensors and microchip-based biological systems for cell biological research, medical research and drug development. The state-of-the-art and future developments, e.g. miniaturization of planar interdigitated electrodes in order to achieve a gap/width size regime on the nanometer scale and thus considerable signal amplification, are summarized [9,10].

Electron transfer by thermally activated hopping through localized centers is an essential element for a broad variety of vital biological and technological processes. The use of electrode/self-assembled monolayer (SAM) assemblies [11] to explore fundamental aspects of long-and

short-range electron exchange between electrodes and redox active molecules, such as proteins, are reviewed comprehensively in a chapter.

In the chapter we provide also a clear overview of the fundamentals and applications of nanoelectrochemistry in biology and medicine. The key concepts related to the double layer, mass transport and electrode kinetics and their dependence on the dimension and geometry of the electrode will be discussed. Next, various fabrication schemes utilized in making nano-sized electrodes are reviewed, along with the inherent challenges in characterizing them accurately [12].

Quantum-dot electrodes [13] and charging, and finite-size effects are also described. Recent advances in the electrochemistry-electrochemical scanning probe techniques used in the investigations of immobilized biomolecules are presented. Finally, a brief survey of the applications of nanoelectrodes in biosensors and biological systems is provided [14].

2. Cellular redox environment — monitoring

2.1. Cellular redox environment — the biological significance

It is now realized that the direction of many cellular processes depends on redox state. But at present the term redox state is not very well defined. The research in this area is mostly observational in that cells or tissues are subjected to an oxidative or reductive stress and then the effects are observed. In general, energy is required to maintain the ordered state of a living organism [15]. The energy comes from the movement of electrons from oxidizable organic molecules to oxygen. This results in an overall reducing environment in cells and tissues. Redox couples in cells are, of course, responsive to electron flow, that is, changes in the reducing/oxidizing environment. Some of these redox couples are linked to each other to form a set of related couples. Sets of couples can be independent from other sets if activation energies for reactions are high and there are no enzyme systems to link them kinetically. The redox environment of a cell is a reflection of the state of these couples [15].

The imbalance of the cellular redox environment is described as oxidative stress which can lead to the damage of biologically important molecules, such as proteins, lipids and DNA. Therefore, it is crucial to detect the cellular redox environment. To achieve this, besides detecting the reactive oxygen species (ROS) which are the indications of oxidative stress, it is also possible to detect the reducing capacity of the cells which plays an inevitable role in keeping the balance of the cellular redox environment.

The original works connected with cellular redox biochemistry were done by Bücher [16]. He developed approaches to determine the states of various redox couples in cells and was the first to estimate the actual cellular reduction potentials (*Ist-Potential*) for the NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ couples.

These days the definition of redox environment should be more general; the redox environment of a linked set of redox couples as found in a biological fluid, organelle, cell, or tissue is

the sum of the products of the reduction potential and reducing capacity of the linked redox couples present [15].

Reduction potential can be thought of as a voltage and reducing capacity would be total charge stored, that is, number of electrons available. Reducing capacity would be estimated by determining the concentration of the reduced species in a redox couple; the reduction potential can be estimated with the *Nernst equation*. In mathematical terms this could be represented by:

$$\text{redox environment} = \sum_{i=1}^{n(\text{couple})} F_i X [\text{reduced species}]_i \quad (1)$$

where, E_i is the half-cell reduction potential for a given redox pair and $[\text{reduced species}]_i$ is the concentration of the reduced species in that redox pair.

It may be difficult (in practical point of view) to measure all linked redox couples present in biological settings to determine the redox environment. Alternatively, a representative redox couple could be used as indicator for changes in the redox environment. For example, in the cell the GSSG/2GSH (glutathione system) couple provides a very large pool of reducing equivalents. It is considered to be the cellular redox buffer. Therefore, the redox state of this couple could be used as an indicator for the redox environment of the cell [15].

Glutathione is considered to be the major thiol-disulfide redox buffer of the cell [17]. On average, the GSH concentration in the cytosol is 1–11 mM [18]. This is far higher than most other redox active compounds. Measurements of total GSH and/or GSSG levels have been used to estimate the redox environment of a cell. Many researchers estimate the redox state of the system by taking the ratio of $[\text{GSH}]/[\text{GSSG}]$. This is convenient as the units divide out, so it is not necessary to determine an absolute concentration. A measurement in mg/mg protein, arbitrary fluorescence units, or the area under an HPLC peak can be entered into the ratio and a useful estimate made. In contrast to the NADPH system however, the absolute concentrations of the components of the GSSG/2GSH redox pair have an impact on the reduction potential. The half-cell reaction is:



Thus, the *Nernst equation* for the reduction potential of the GSSG/2GSH half-cell will have the form:

$$E_{\text{hc}} = -240 - (59.1/2) \log \left([\text{GSH}]^2 / [\text{GSSG}] \right) \text{ mV} \quad (3)$$

at 25°C, pH 7.0

A good example for the importance of calculating E_{hc} rather than estimating only the GSH/GSSG ratio, is provided by Kirilin et al. [19]. Colon cancer cells were differentiated with sodium butyrate and then exposed to benzene isothiocyanate, a compound that induces detoxifying enzymes. Sodium butyrate brought about a 3.6-fold decrease in the GSH to GSSG ratio; addition of benzene isothiocyanate decreased the ratio 3.7-fold. Sodium butyrate changed E_{hc} by 14 mV while benzene isothiocyanate changed E_{hc} by 40 mV. The difference of 26 mV in E_{hc} resulted because when cells were treated with benzene isothiocyanate they had a much smaller GSH pool due to the pretreatment with sodium butyrate.

Development of an advanced organism starts with the division of a single cell. The progeny of this cell must turn into a multi-cell, multi-tissue, and multi-functional entity. This development occurs in a relatively reducing environment.

In eukaryotes, cells divide when they attain a certain size or when triggered by extracellular stimuli such as growth factors or hormones. Proliferative signals flow through intracellular signaling pathways to activate the cell-cycle (Figure 1).

Stress signals from within or outside a cell oppose proliferation. Therefore, cells rely on proliferative signaling pathways, as well as stress surveillance systems (or checkpoints), to regulate entry into the cell-cycle [20, 21]. Stress signaling, particularly in response to DNA damage and infection, is vital for survival to ensure cells elicit appropriate defense or repair mechanisms [22].

The signaling cascades triggered by DNA damage and inflammatory responses have been well studied to this moment [20]. In addition to the primary damage, many stress situations induce reactive oxygen species (ROS). ROS are highly reactive radicals or molecules produced intracellularly from several compartments (mitochondria, endoplasmic reticulum, peroxisomes) [23]. However, they are also induced by external sources such as ionizing agents, vitamins, or herbicides [24].

ROS can interact with biomolecules resulting in oxidation of amino acid residues in proteins, mutations in DNA, and lipid peroxidation producing more free radicals. Excess production of ROS in cells overwhelms cellular detoxifying systems, resulting in oxidative stress [24].

Recently, an increasing body of work has indicated that redox signaling is mediated by switching the redox state of certain cysteine residues in proteins to elicit an oxidative stress response in cells [25]. Although reactive cysteine residues have been identified in many cell-cycle regulators, their function in controlling cell division has only recently been elucidated in yeast [26,27].

Redox regulation by specific cysteine residues opens up the possibility of manipulating cell division by designing redox-active molecules to block the activity of cell-cycle regulators.

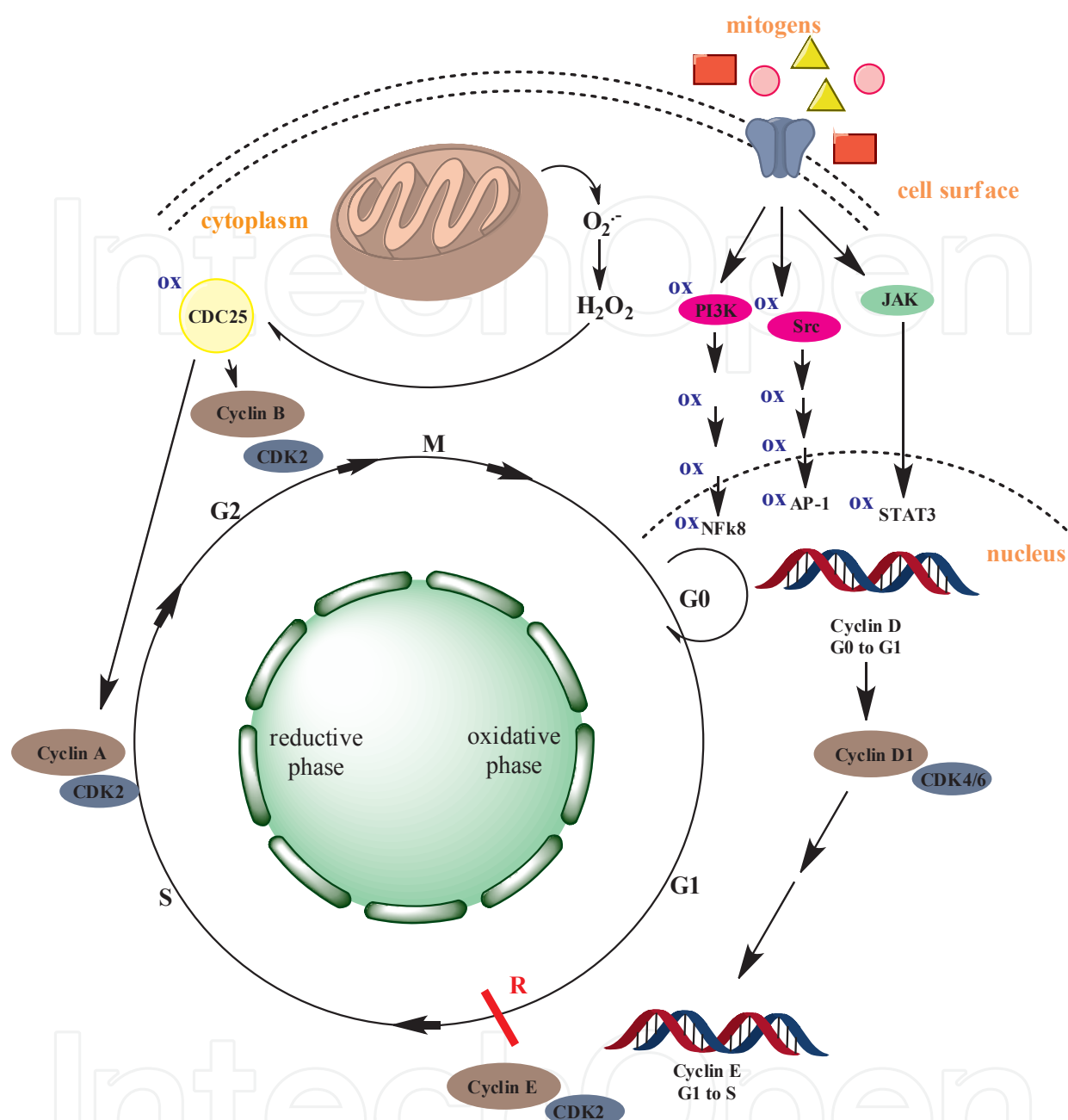


Figure 1. Overview of redox control of the eukaryotic cell-cycle. ROS (i.e. superoxide and H₂O₂) are produced as a result of oxidative phosphorylation in the mitochondria. Cell-cycle regulators with reactive cysteine residues are highlighted by a dark blue 'ox'. In response to mitogens, signaling pathway kinases convey proliferative signals to activate expression of cyclin D. Cyclin D complexes with Cdk4 or Cdk6 and phosphorylates to release its inhibitory effects driving cells to re-enter the cell-cycle from quiescence to G1. Once the restrictive point at late G1 (labeled as red R) is passed, cells are committed to cell division. Activation process leads to the transcription of cyclin E for transition from G1 to S phase. Subsequent expression of cyclin A and cyclin B leads to transition of S to G2 and G2 to M phases, respectively. The phosphatase Cdc25 activates cyclin A–Cdk1, cyclin B–Cdk1, cyclin E–Cdk2 for entry into M phase by removing the inhibitory phosphorylation on Cdk1 and Cdk2. The cell-cycle is synchronized with the metabolic cycle of the cells with S phase and M phase occurring only during the reductive phase of metabolism and G1 in the oxidative phase [according to 20].

2.2. Signaling through cysteine modification

Signals transduced by phosphorylation relays are amplified to produce a prolonged response in cells. This is beneficial during proliferation to ensure cellular activities are diverted to cell division and growth in response to growth stimuli [28,29].

Similarly, it would be advantageous for cells to amplify signals from harmful stimuli to remove relatively stable damage. A burst of ROS usually occurs in a localized manner and is short-lived. Therefore, redox signaling uses a different mechanism for sensing through reactions with specific residues in target proteins to sense and transmit signals [30].

In other words, the 'receptors' of ROS are specific reactive groups, mainly thiol groups of highly reactive cysteine residue(s) within targeted proteins [30]. Cysteine residues are widespread in proteins – the specificity of the 'ROS receptors' in redox signaling is determined by the susceptibility of cysteine residues to oxidation, which is determined by the pKa of their thiol groups [31]. In free cysteine, this pKa is approximately 8.2 [31], but cysteine residues located in proximity to positively charged basic residues, aromatic residues, or metal centers have lowered pKa (<6.5).

Depending also on the accessibility of a cysteine thiol to the solvent, its pKa can be influenced by small fluctuations in the local pH [32]. The micro environment of a cysteine residue therefore determines its reactivity. After oxidation, cysteine residues can be modified (Figure 2) by: formation of intramolecular or intermolecular disulfide bonds; formation of mixed disulfides with glutathione; and oxidation of the cysteine thiol (R-SH) to sulfenic acid (R-SOH) or to the more highly oxidized sulfinic (R-SO₂H) or sulfonic (R-SO₃H) acids [23]. When a vicinal cysteine for disulfide bond formation is not available, sulfenic acid intermediate can also react with the main chain nitrogen of an adjacent residue forming a sulfonamide.

All these modifications have been observed in cell-cycle regulators in response to oxidative stress. Although oxidation of cell-cycle regulators has been detected *in vitro* in many cases [34], more recently, an increasing number have also been demonstrated *in vivo* [20, 36].

2.3. Monitoring of cellular redox environment-methods

The intracellular redox environment is a highly dynamic arrangement governed by the formation and degradation of many reactive species of oxygen and nitrogen. Under physiological conditions, the cytosol, the nucleus, and the mitochondrial matrix space maintain homeostatic conditions preferring a highly reducing environment [35]. Intracellular reducing conditions are largely maintained by mM concentrations of reduced glutathione and its enzymes that together constitute the glutathione system [36].

Recent methodological advances have made it possible to focus studies of pro-oxidative changes to specific redox couples within defined subcellular compartments [37], potentially granting greater specificity in mechanistic investigations of the oxidative effects of xenobiotic exposures. A new generation of genetically encoded fluorophores permits direct assessment of the oxidative effects of xenobiotic compounds in relation to the GSH/GSSG redox pair with unprecedented spatial and temporal resolution [37]. Redox-sensitive green fluorescent protein

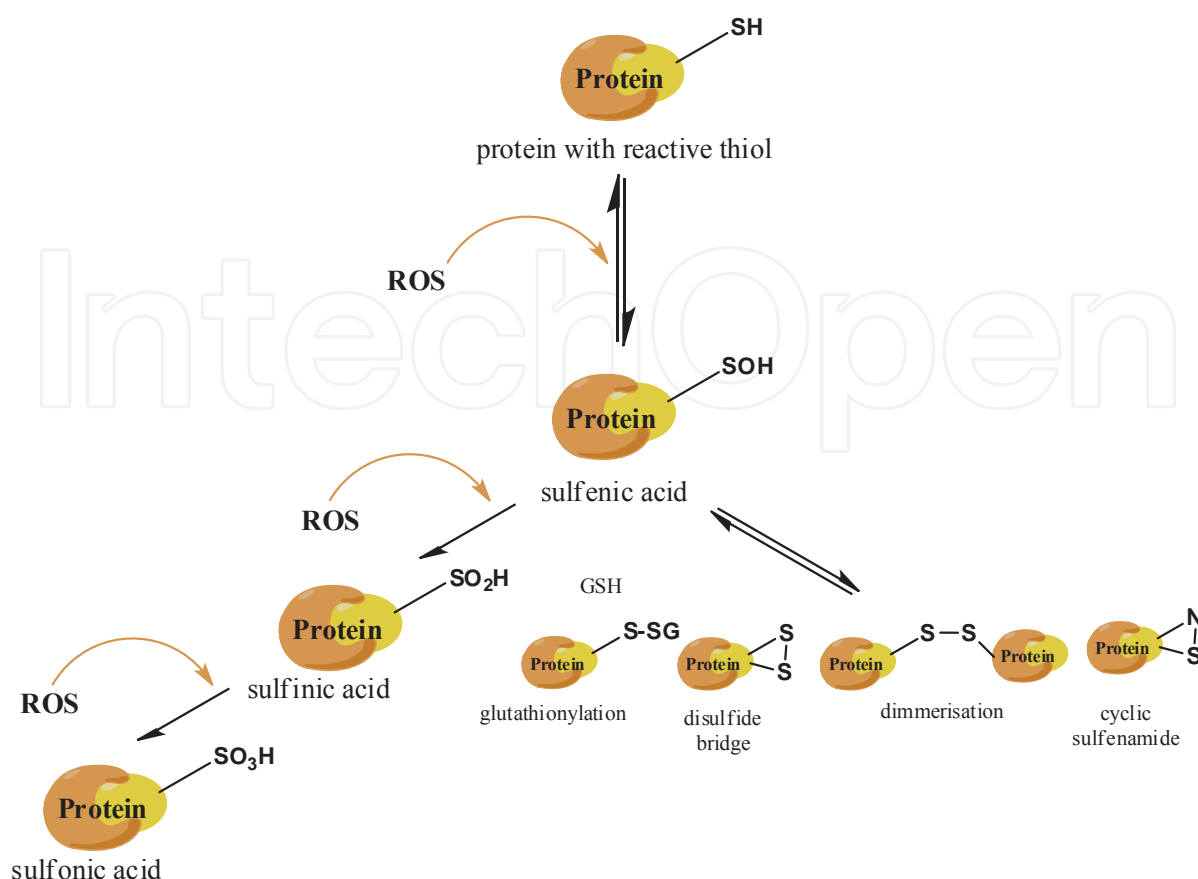


Figure 2. Oxidative modifications of reactive cysteine thiols. Oxidation of a reactive thiol to a sulfenic acid represents the first step for consecutive modification. Reversible modifications of sulfenic acid residues are shown that include: coupling with GSH (S-glutathionylation), disulfide formation with a neighboring cysteine (intramolecular) or with another protein (dimerisation), and covalent linkage to the nitrogen atom of a neighboring amino acid residue to form a sulfenamide. These modifications can be reversed by cellular reductants such as a glutathione (GSH). Sulfenic acid can also be irreversibly oxidized further to sulfinic or sulfonic acid species.

roGFP acts as a reporter of intracellular E_{GSH} by equilibrating with the GSH/GSSG redox pair [37]. In reaction that depends on catalysis by glutaredoxins, roGFP responds to oxidation of reduced glutathione (GSH) to its oxidized form (GSSG) *via* the internal formation of a disulfide bond [37,38] (Figure 3). The formation of the disulfide bond alters the spectral characteristics of the GFP fluorophore causing the intensity of the emitted green fluorescence (~ 520 nm) by excitation at 488 nm to decrease, while causing the emitted fluorescence after excitation at 405 nm to increase, thus making this sensing unit a ratiometric probe. Further efforts to improve the responsiveness of roGFP have led to the conjugation of pathway-specific enzymes to create a fusion of proteins operating as redox relays. In particular, the conjugation of glutaredoxin (Grx) to roGFP has been shown to enhance the kinetics of the roGFP response to the oxidation of glutathione [38].

Fluorescent probes for detection of the cellular redox environment are promising tools that can provide spatial and temporal information in living cells. An excellent probe is to couple the nitroxide with a fluorophore covalently (F-NO[•]). The fluorescence of the fluorophore can

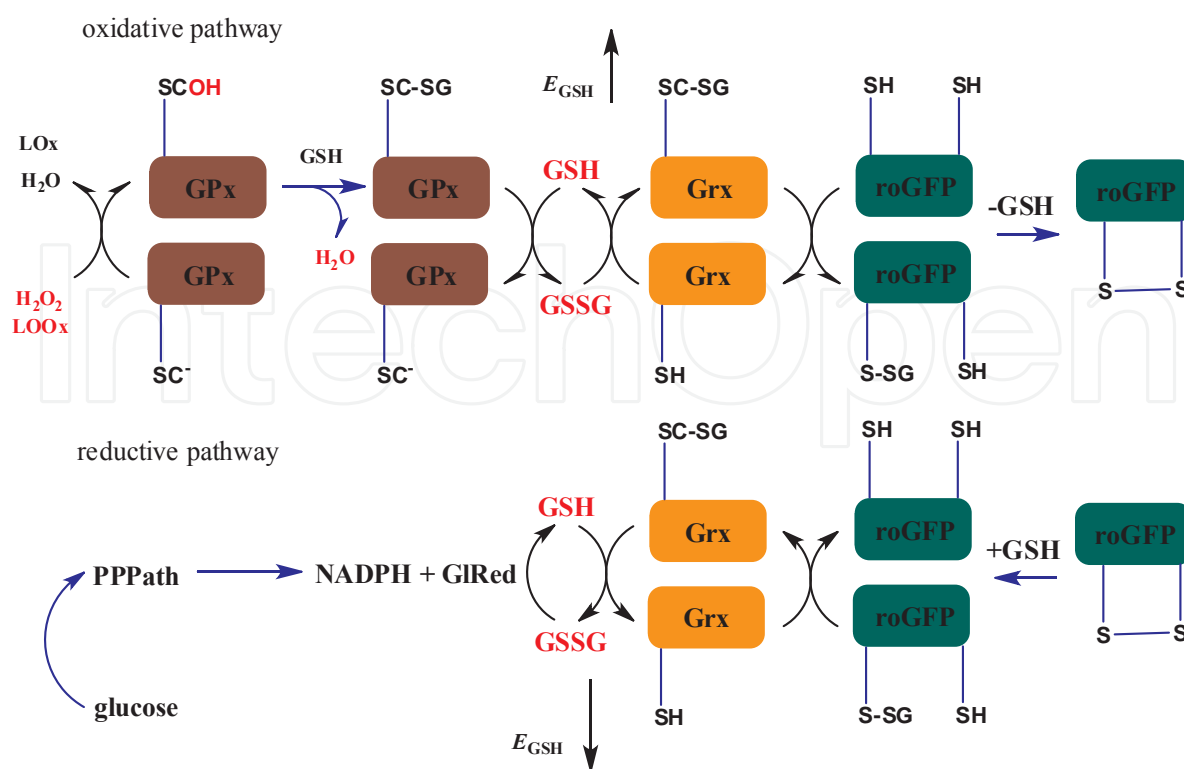


Figure 3. roGFP interactions with the glutathione system (according to [38]). Glutathione peroxidases (GPx) oxidize GSH to GSSG in response to peroxides, including H_2O_2 and lipid hydroperoxides (LOOx), thus increasing the glutathione redox potential (E_{GSH}). Abbreviations: LOx, reduced lipid oxide; SC^- , reduced selenocysteine; SCOH, oxidized selenocysteine; SC-SG, glutathionylated selenocysteine. In response to the increase in GSSG, one of the engineered vicinal cysteines of roGFP becomes S-glutathionylated by glutaredoxin (Grx). Glutathionylation in turn causes disulfide bond formation and alteration of the spectral properties of the GFP fluorophore. In the reductive pathway, Grx catalyzes the reduction of roGFP disulfide bonds through deglutathionylation as GSSG levels decrease and normal levels of GSH are reestablished by glutathione reductase (GRed), at the expense of NADPH, causing a renormalization of E_{GSH} . Glucose and the pentose-phosphate pathway (PPPath) create NADPH, which is used by GRed to reduce GSSG to GSH.

be quenched efficiently by the nitroxide through electron exchanges but recovers significantly when converting to the corresponding diamagnetic derivatives by reduction or reaction with a free radical [39,40]. So it is usually called a profluorescent probe. The probes have been used to detect free radicals [41], ascorbic acid [42] and the redox environment of the cells [43]. The nitroxide reduction is mediated by various reductants, such as ascorbic acid, GSH (glutathione), NADPH and ubiquinol [34]. However, in cytosol, the reduction of nitroxide is primarily due to ascorbic acid, especially in hepatic cells where the concentration of ascorbic acid is higher than in the other cells [45]. So it is of potential to monitor the redox environment of hepatic cells by F-NO \cdot probes based on the reduction of the nitroxide.

Expanding evidence suggests a close relation between glucose metabolism and cancerogenity. The tumour cells metabolize glucose more fiercely than normal cells and the apparent difference has led to attempts to develop novel cancer therapeutic strategies [46].

Despite the fact, the advantages of selective redox-regulating systems for cancer therapy have been in considerable attention, a method for testing the therapy effects of the regulators remains imperceptible, it is difficult to measure the intracellular redox environment over time.

One of the method probe to monitor the redox environment is synthesized by Cao et al. [47] a novel rhodamine nitroxide probe to observe the redox environment of human hepatoma cells and normal human liver cells by detecting the reducing capacity of the cells. The synthetic route and reduction mechanism are shown in Figure 4.

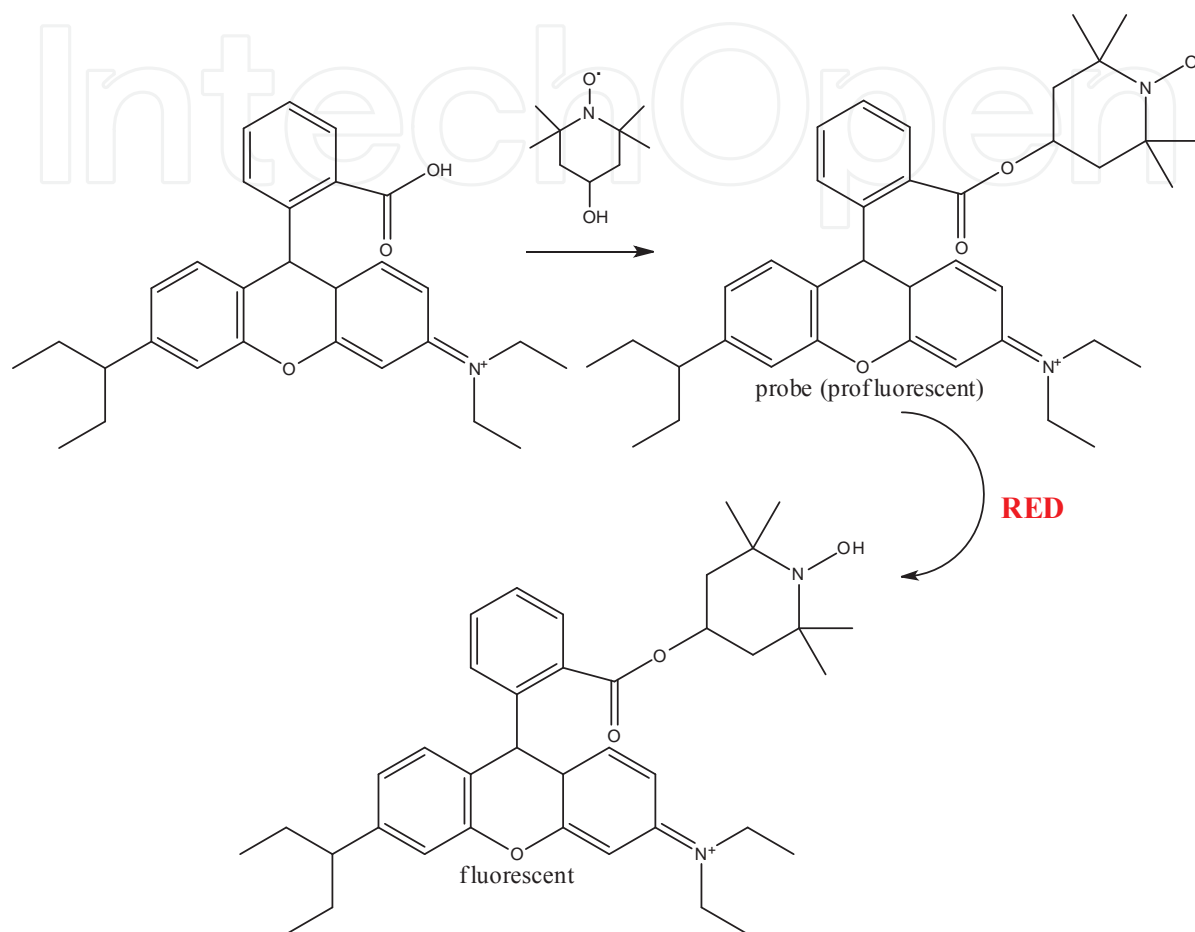


Figure 4. The synthetic route and reducing mechanism of the probe (novel rhodamine nitroxide)

Several other typical biological reducing compounds were also selected to verify the reduction properties of the probe for further application in cells. The addition of these reducing compounds, namely GSH, cysteine and uric acid, resulted in a small increase of the fluorescence.

3. Exocytosis — monitoring

3.1. Exocytosis — function

Exocytosis is a general term used to denote vesicle fusion at the plasma membrane, and it is the final step in the secretory pathway that typically begins in the endoplasmic reticulum, passes through the Golgi apparatus, and ends at the outside of the cell. Endocytosis refers to

the recovery of vesicles from the plasma membrane (Fig. 4). Exocytotic vesicle fusion involves the coalescence of vesicle and plasma membranes [48] and allows the so-called fusion pore to form. The fusion pore is a channel that passes through the vesicle and plasma membranes and allows delivery of the vesicle contents to the extracellular compartment. Docking is the process by which the exocytotic vesicle is fixed beneath the plasma membrane before fusion. It is generally believed to involve molecular recognition between vesicle and plasma membrane and is therefore one aspect of vesicle targeting. Another kind of targeting can be provided by the cytoskeletal proteins that move vesicles around the cell [48].

A literature review indicated that there are at least three parallel routes from the Golgi apparatus to the cell surface in mammals and that these pathways operate to varying extents in different cell types [49]. There is no reason to suppose that in plants the situation is any less complex. It cannot be determined by inspection whether vesicles are exocytotic, endocytotic, or bound for destinations other than the plasma membrane. In many cases, however, particularly in green algae [50], it seems clear that there is more than one type of vesicle delivered to the plasma membrane. Electrophysiological data similarly indicate at least two vesicle populations in barley aleurone protoplasts [51].

The commonly held view is that secretory vesicles are delivered to their target membrane by the cytoskeleton. In animal cells, this delivery role is effected by microtubules [52], and vesicles are distributed from a centrally located Golgi apparatus to the plasma membrane. In plants, there are many Golgi apparatuses in each cell, and microfilaments play the major role in vesicle delivery, as is evidenced in highly polarized cells such as the pollen tube, where microfilaments must transport vesicles considerable distances [53]. Microtubules are also present but appear to be more concerned with nuclear migration and cytoplasmic area than vesicle transport [54]. Even in cells from the coleoptile and root, which exhibit less polarized growth, current evidence suggests a mainly microfilament-based vesicle transport mechanism [55].

Proteins that have a putative protective function often exemplify post-Golgi processing and maturation [56]. In many cases, these proteins accumulate within vacuoles and presumably are released by holocrine secretion, a crude type of exocytosis in as much as it is characterized by the release of vacuolar contents coupled with cell degeneration. As an example, a proteinase inhibitor that is believed to act against insects [57] is expressed in stigmatic cells of *Nicotiana glauca* as a pentameric protein that is thought to undergo conversion to its active form within the vacuole [58]. On the assumption that the protein precursor is inactive, it has been suggested that such post-Golgi activation may provide a self-protection mechanism. Proteins with putative antimicrobial activity, such as 2S albumins, thionins, and chitin binding proteins, also accumulate within vacuoles, and their release can involve non-specific cell breakdown. Nevertheless, the release of vacuolar contents in response to invasion and other triggering processes may be more regulated [59].

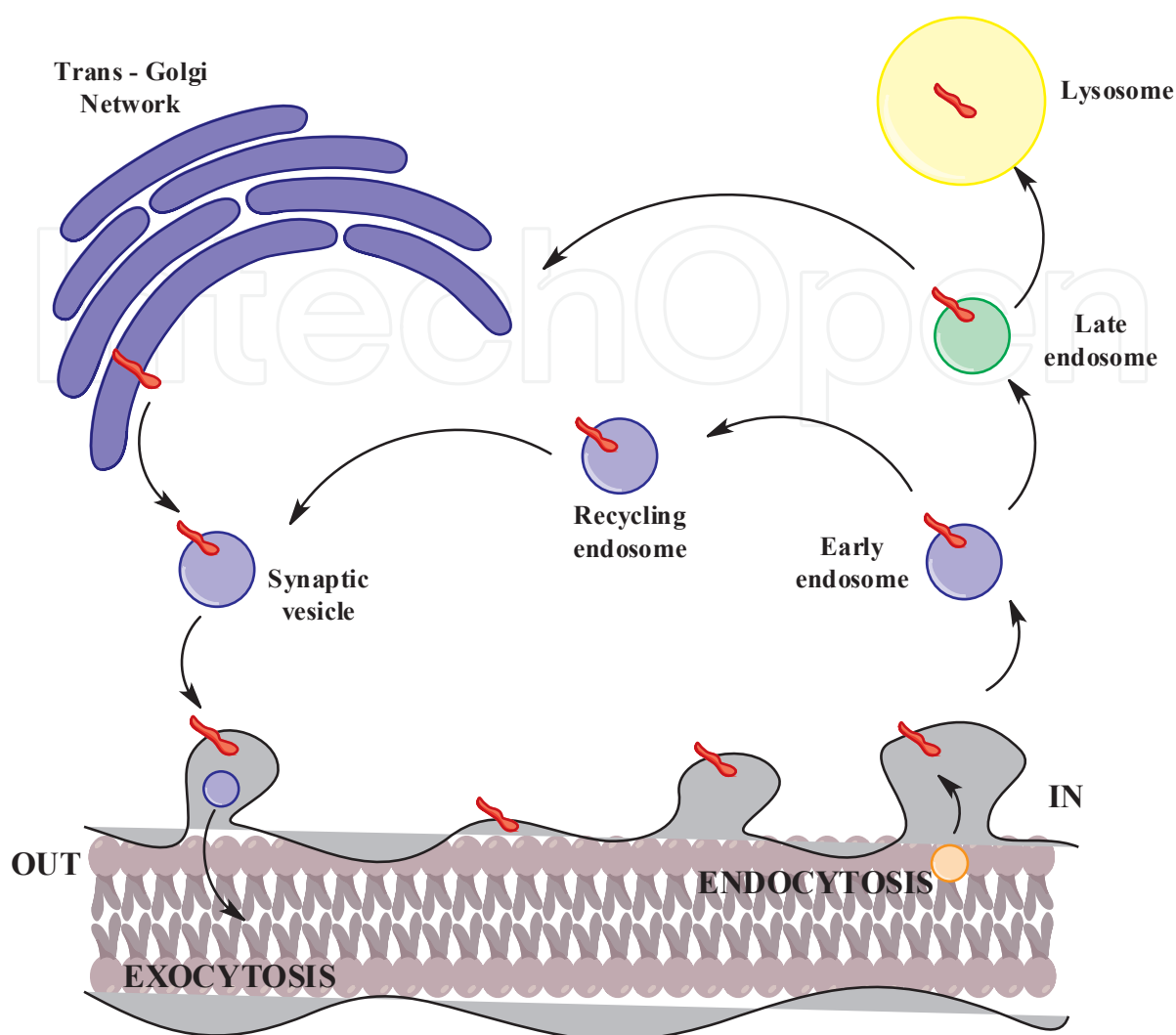


Figure 5. Scheme of exo-and endocytosis

3.2. Exocytosis — methods of monitoring — electrochemistry

Intercellular communication occurs through the release of chemical (or biochemical) messengers from an emitting cell to a target cell. This transmission is mostly achieved by vesicular exocytosis, such as in neurons, neuroendocrine cells, or for the control of hormonal fluxes in blood [60]. The expulsion of the messengers from the emitting cell and their further diffusion toward the neighboring cells can be briefly shown. First of all, secretory vesicles located in the emitting cell cytoplasm are initially filled with the biochemical messengers [61]. Second, following an appropriate cell stimulation (which provokes a Ca^{2+} entry or increase), the available vesicles dock to the cell membrane through a step requiring multiple protein-protein interactions, that is, the formation of soluble *N*-ethylmaleimide sensitive fusion protein attachment receptors complexes [62]. To overcome the natural electrostatic repulsions between the cell and vesicular membranes, *N*-ethylmaleimide sensitive fusion protein attachment receptors complexes allow membranes to interact at molecular distances so that the electrical

field carried by each electroporates them. According to local reorganization of phospholipidic bilayers, the formation of a fusion pore ensues through which the release of the vesicular content toward the extracellular medium onsets. In most cell models investigated experimentally, exocytosis implies “dense-core” vesicles, whose intrinsic properties make them a better analytical target. Thus, the cationic messengers are compacted into a matrix behaving like a polyelectrolytic gel and constituted of polyanionic proteins such as chromogranins, which fill the vesicle core. Because of the formation of the fusion pore, the first ionic exchanges between this matrix and the extracellular medium occur spontaneously by allowing partition of the cations. This situation provokes a local exchange with the cations of the external medium and thus a destructure of the gel due to the different molecular and supramolecular interactions [60]. In a natural environment, each messenger cation is exchanged by a fully hydrated small cation (Na^+ , H_3O^+), and a local matrix swelling is induced. This increases the *Laplace tension* over the membranes junction area, which may ultimately counterbalance the pore edge energy.

3.2.1. Fast scan cyclic voltammetry and amperometry: respective advantages

Aerobic living cells require oxygen-containing solutions to perform as close as possible to physiological conditions. Because O_2 is in high or comparable concentrations (0.23 mM) *vs* the released species flowing into the semi artificial synapse, reductive detection is generally prohibited [8]. Electrochemical measurements of a cell secretion then generally rely on the oxidation of the molecules released at the electrode surface located close to the cell membrane. The recorded currents evidence the nature of the molecules released and their quantities as well as the dynamics of the release itself. Two main electrochemical techniques are applied, amperometry and cyclic voltammetry [7,8].

In amperometry, the working electrode is held at a constant potential at which the molecules under investigation are oxidizable. It ensues a continuous recording of the oxidation current as a function of the time without severe contamination by capacitive current. However, the “potential information” is lost.

In cyclic voltammetry, the current is recorded as a function of the potential applied at the electrode (triangular voltage ramps), so discontinuous monitoring ensues, and capacitive currents may impede measurements, whenever they are not stable enough to be subtracted. In the absence of a semi-artificial synapse configuration, electrochemistry is coupled to another analytical technique (liquid chromatography or capillary electrophoresis) [8].

Unfortunately, although such measurements may be achieved at the single cell level, they merely report confirmations once the whole exocytotic process has ended [63]. As example, the catecholamines content of a single bovine chromaffin cell can be determined by analyzing the extracellular solution (resulting from the cell lysis or after exocytosis) through microcolumn liquid chromatography coupled with amperometry at a carbon fiber electrode [64]. This type of study has allowed the quantification of the amount of catecholamines per cell, and it has further evidence that chromaffin cells are classified in either a single type of catecholamines secreted or a mixture of species released. Moreover, the individual cells have been shown to deliver catecholamines in the same proportions in which they store catecholamines [65]. Similar studies (in which the coupled electrochemical method is voltammetry) were also

performed on individual neurons [66]. Although these postanalytical methods can provide essential data concerning the vesicular content, no temporal and spatial information could be obtained during the very exocytotic release system. In the semi-artificial synapse mode, amperometry or voltammetry can be achieved along the occurrence of effective exocytotic events. As example, successive cyclic voltammograms performed on chromaffin cells at relatively low scan rates (10 Vs^{-1}) during exocytosis afforded a clear discrimination between adrenaline and nor-adrenaline releases. From the voltammogram of catecholamines under physiologically compatible conditions, an oxidation of the catechol into *o*-quinone during the anodic scan and subsequent reduction of the latter during the reverse cathodic scan can be observed [61]. An additional peak is detected on the reverse reduction scan for adrenaline, due to a fast cyclization reaction that eventually leads to the formation of a reducible adrenochrome, which is reduced [65].

After appropriate stimulation, vesicles that are primed to undergo exocytosis dock to the cell membrane by the mean of *N*-ethylmaleimide sensitive fusion protein attachment receptors complexes. The cell and vesicular membranes mix and evolve to the formation of a fusion pore, through which neurotransmitters begin to diffuse out the vesicular matrix. Resulting from the ionic exchanges (catecholamines cations vs Na^+ or H_3O^+) and water entry between the intravesicular and the extracellular media, the vesicular matrix swells and provokes the fusion pore expansion.

Voltammetric studies performed during the exocytotic release by a single living cell could thus confirm the results obtained with chromatography, that is, at least three classes of chromaffin cells (one that only releases adrenaline, a second one that only releases nor-adrenaline, and a last one that releases a mixture of adrenaline and nor-adrenaline) may be implied during catecholamines secretion in adrenal glands [65].

It was found also that, the fast scan voltammetry (usually 800 Vs^{-1}) also allows the continuous differentiation of adrenaline and nor-adrenaline.

As compared to cyclic voltammetry measurements, complex features of the dynamics of the exocytotic events can be amperometrically obtained.

In general, four main parameters of the amperometric trace have been evidenced to reveal the features of secretion events at a single cell. The first one deals with the number of events detected per cell (frequency of release), and the others concern the characteristics of the individual amperometric spikes and provide individual and statistical data about the progress of the event itself [60].

4. Mechanisms of electron exchange between electrode and protein

Electrochemical methods offer several well-recognized, classical advantages over homogeneous approaches for elucidating charge transfer processes. Two of the more important advantages are:

- the ability to study a single redox-active component by using an electrode to act as the other reactant [67-68];
- and the ability to gradually differ the redox potential (electrode overvoltage); this trait allows for the gentle variation of the reaction free energy, ΔG_0 [67-68]. Standard heterogeneous pathways also have some obvious disadvantages. Noteworthy are the strong impact of the electrode/solution interfacial potential drop on the electron transfer process, especially in the free diffusion regime for which it complicates the over-all kinetic analysis [68,69]; and possible changes in the redox-active molecules upon adsorption to an electrode; in particular the adsorption of biomacromolecules, such as proteins, can lead to their inactivation [69].

The above mentioned disadvantages can be largely eliminated through self-assembly techniques that coat the electrode with a biocompatible surface. One common method uses thiolate groups, which form weak covalent bonds with surface-exposed atoms of metal electrodes; e.g., Au, Ag, Hg, etc. [70], and lead to the formation of almost perfect (defect-free) *quasi*-crystalline self-assembled monolayers – SAMs films. The chemical and physical properties of these films can be modified to provide additional control over the electron transfer. These include the variation of the chemical identity and composition (mixing/dilution) of the SAM terminal groups that are presented to the solution (electrolyte); e.g., they can be manipulated to control the binding position and orientation of biomolecules [69].

4.1. Freely diffusing redox couple — electron transfer

Systematic studies of electron exchange for freely diffusing redox couples with SAM-coated metal electrodes show a similar dependence of the rate constant on the SAM thickness. The most systematic studies of the couples $\text{Fe}(\text{CN})_6^{3-/4-}$ [71] and $\text{Ru}(\text{NH}_3)_6^{3+/2+}$, [72] at Au/SAM junctions show that the nature of the redox couple including the charge type and distribution within the ligand sphere plays an important role in determining whether water and counterions can penetrate into the SAM interior or be almost ideally excluded from the SAM (the case of $\text{Fe}(\text{CN})_6^{3-/4-}$).

The results [73] indicate that the kinetically fast redox couple $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ is better able to penetrate into unicomponent, CH_3 -terminated SAMs of the general type: $\text{S}-(\text{CH}_2)_n-\text{CH}_3$, with n running from 2 to 18, than is $\text{Fe}(\text{CN})_6^{3-/4-}$, and thus it can detect small defects in the SAM.

This interpretation has been substantiated by theoretical calculations of the charge distribution within the complex ions, which demonstrate that hexamine metal complexes have most of the excess charge located on the metal ion core, whereas hexacyano-metal complexes have it localized on the terminal nitrogen atoms of cyano-ligands [74]. This valid difference suggests that $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ species are able to penetrate more readily into SAMs and diffuse along the SAM chains, in the presence (or even absence) of probable collapsed sites, as compared to $[\text{Fe}(\text{CN})_6]^{3-/4-}$. This difference could cause more variation in the effective charge-transfer distance, as compared to the ideal case with impermeable SAMs. Moreover, it can be supposed that, the SAM defects can be roughly classified as static and dynamic ones, ascribing the former to different kinds of pinholes and collapsed sites, whereas the latter corresponds to increased mobility, and may or may not be associated with particular structural defects [69].

These findings suggest that the dynamically controlled mechanism may be more pronounced in slowly relaxing media such as bio-molecules, ionic liquids or liquid crystals.

Indeed, the interior and the SAM- and solvent-adjacent layers of proteins can be viewed as an extremely viscous media with characteristic dielectric relaxation times similar to pure glycerol [75-76].

4.2. SAM — modified electrodes and immobilized species

The development of SAM-coated electrodes has enabled fundamental and applied studies of biomolecular electron transfer mechanisms, and offers many promising opportunities for creating arrays of redox active biomolecules. As methodologies to immobilize proteins [77-78] improve so that it is possible to better control over protein orientation and spatial placement on an electrode surface, the detail and precision of fundamental questions that can be addressed will improve and the applications of electrically wired protein arrays will increase. According to literature, applying of electrode/SAM assemblies to explore fundamental aspects of the electron exchange between electrodes and redox active molecules; most particularly, proteins is extremely interested field. These new developments identify a need to generalize modern charge transfer theory and bear on the use of electrochemical methods in modern bioengineering and biomedical applications [79]. Even the very first applications of SAM-based electrochemical systems enabled fundamental testing of basic features of contemporary charge transfer theory [69].

For example, studies of the electron transfer rate as a function of SAM thickness revealed that the nonadiabatic (diabatic) mechanism, or electron tunneling, is the rate controlling step for long range electron transfer. In this limiting case, theoretical considerations predict a distinct experimental signature, an exponential decay of the electron transfer rate constant, k_{et} , with the distance:

$$k_{ET(NA)} \propto \exp [-\beta(R_e - R_o)] \quad (4)$$

where R_e is the electron transfer distance, R_o is the electron transfer distance at the closest approach of the redox-active couple to the electrode, and β is the decay parameter, normally of the order of *ca.* 1 \AA^{-1} [69].

Until recently, the mechanism(s) controlling short-range electron transfer, either at bare conductive electrodes (mostly implying small redox species), or SAM-modified metal electrodes (simple model systems as well as redox-active biomolecules), has remained more controversial. A generalized version of the classical electron transfer theory [69] accounts for a change in the inherent reaction mechanism with a gradual increase of electrode-reactant electronic coupling. The model systems allow the coupling to be tuned by changing the SAM thickness from thick (weak coupling/long range of electron transfer) to thin (strong coupling/short range of electron transfer) and in essence explore the connection between different electron transfer limits [80].

A number of examples of rigorously confirmed conformationally gated electron transfer have been reported [81] and it appears to be of considerable biological importance. Moreover, conformational gating should be considered as a special case compared to the more classical adiabatic electron transfer mechanism that has an essentially universal origin [79]. Unfortunately, as far as biological electron transfer is concerned, except of several cases limited to different cytochrome c assemblies [82,83] and azurin [84], the friction controlled mechanism has not been discussed in detail. In fact, viscosity-dependent kinetics (exhibiting a weak dependence of the rate on the SAM thickness) is often presented as arising from a conformational-gating mechanism without relevant experimental cross-testing or analysis to ascertain whether it could arise from a friction controlled electron transfer mechanism.

5. Nanomaterials and biosensors

The advantages and new possibilities offered by nanotechnology are varied. Materials exhibit new properties when scaled down from bulk material to nanometric dimensions. These properties can be precisely fine tuned, thus allowing for the fabrication of defined structures and materials optimized for a certain purpose.

Consequently, nanomaterials and concepts from nanotechnology have been much employed in biosensor development. Several reviews in this field [85-88] provide a distinct overview of the possibilities of nanotechnology in the field of biosensor research. The following summarizes the most important trends. The main challenges in the application of nanomaterials for biosensor designs are the definition of the material features, the reproducible synthesis of materials with suitable properties, and the meaningful application of nanotechnological concepts to biosensors. Instead, multidisciplinary effort will be necessary to obtain nanomaterials with properties as required for a novel biosensor design. The seemingly most challenging task of applying nanotechnology to biosensors is to really make use of “nano features” and not simply using nanomaterials without them adding value to the biosensor architecture. In the area of biosensor research some features of nanostructures become important in addition to pure material properties [1,2]. For example, in nanometric structures diffusion lengths become very short and hence mass transport is highly efficient. Since mass transport is valid in many biosensor designs, an increase or at least a change in sensor performance can be expected from using nanometric structures [8]. There are basically three broad categories of pathways towards nanobiosensors and in particular in electrochemical nanobiosensor development. The modification of a macroscopic transducer with nanomaterials is the first of these approaches. In electrochemical biosensors, this would translate into large electrodes modified with nanomaterials. The second approach is the miniaturization of the transducer, namely the use of nanoelectrodes [89] or other miniaturized circuitry of nanometric dimensions [7].

The modification of biomolecules with nanomaterials or coupling of biomolecules and nanomaterials is the third category of approach towards nanobiosensors. Of course, the lines

between these approaches are blurred and some sensor designs may draw from more than one of these concepts.

5.1. Modification of biomolecules with nanomaterials

The oriented modification of biomolecules with nanomaterials resulting in biomolecule – nanomaterial hybrids offers crucial possibilities for biosensing. Gold nanoparticles can be used to immobilize biocatalysts or other bioelements on electrodes or other supports. However, in this case, the nanoparticles often just function as a linker and the sensor architecture does not benefit from a unique property due to nanometric dimensions. Unlike the unique optical properties of quantum dots (QDs) make these materials well suited as fluorescent labels in optical sensors [90,91] really taking advantage of a nanosized feature. The use of nanoparticle–enzyme hybrids has been recently reviewed [92,93] as has been the use of nanotechnology in the manipulation of redox systems at an earlier stage [94].

Such an establishment of electrical contact between a redox enzyme and an electrode can also be achieved by nanoparticles. Standing out in this field of research is the wiring of redox enzymes by gold nanoparticles [95]. Due to the fact, glucose oxidase (GOx) was reconstituted with a gold nanoparticle (1.4 nm in diameter, corresponding to the size of the redox center of the enzyme) that was functionalized with the enzyme's cofactor FAD [96].

Such enzyme – nanoparticle hybrids were assembled on gold electrodes leading to relevant good electrical contact between the protein redox center and the macroscopic electrode surface.

Nanotechnology has contributed significantly to recent developments in biosensor research. Modification of macroelectrodes with nanomaterials has resulted in new electron transfer properties and biocompatibility. Nanometric transducers have been used to obtain new classes of biosensor devices. Finally, biomolecule – nanoparticle conjugates show a suitable application potential in biodevice development. An aspect of nanotechnology that is rarely mentioned is the potential harm of nanomaterials towards health. Though a significant effort has been put into the research of this field [95,97-98], the consequences of the use of nanomaterials in everyday life are not yet clarified. Therefore, it appears, that the potential risks of nanomaterials are by far outweighed by the possibilities offered by nanotechnology.

5.2. Implanted biosensors in medicine

As shown in Figure 5, (electrochemical) biosensors are either placed in laboratory animals for fundamental physiological and neurochemical *in vivo* measurements or implanted in the human body for health and metabolite monitoring. In the field of *in vivo* medical research, biocatalyst-based analytical tools are often used for spatially confined measurements of their corresponding target species in preselected regions of living test subjects [1].

The vital challenges/problems in the field of implantable sensors are the stability of the device, the selectivity of the sensor, and the biocompatibility of the sensor. Most important, the sensor has not be rejected by the living organism. When implanted, the sensor should operate for a prolonged time to justify any surgical procedure necessary for the introduction of the sensor

into the body. Even when these two challenges are ensured, the sensor has typically to deal with a very complex sample matrix, most commonly blood.

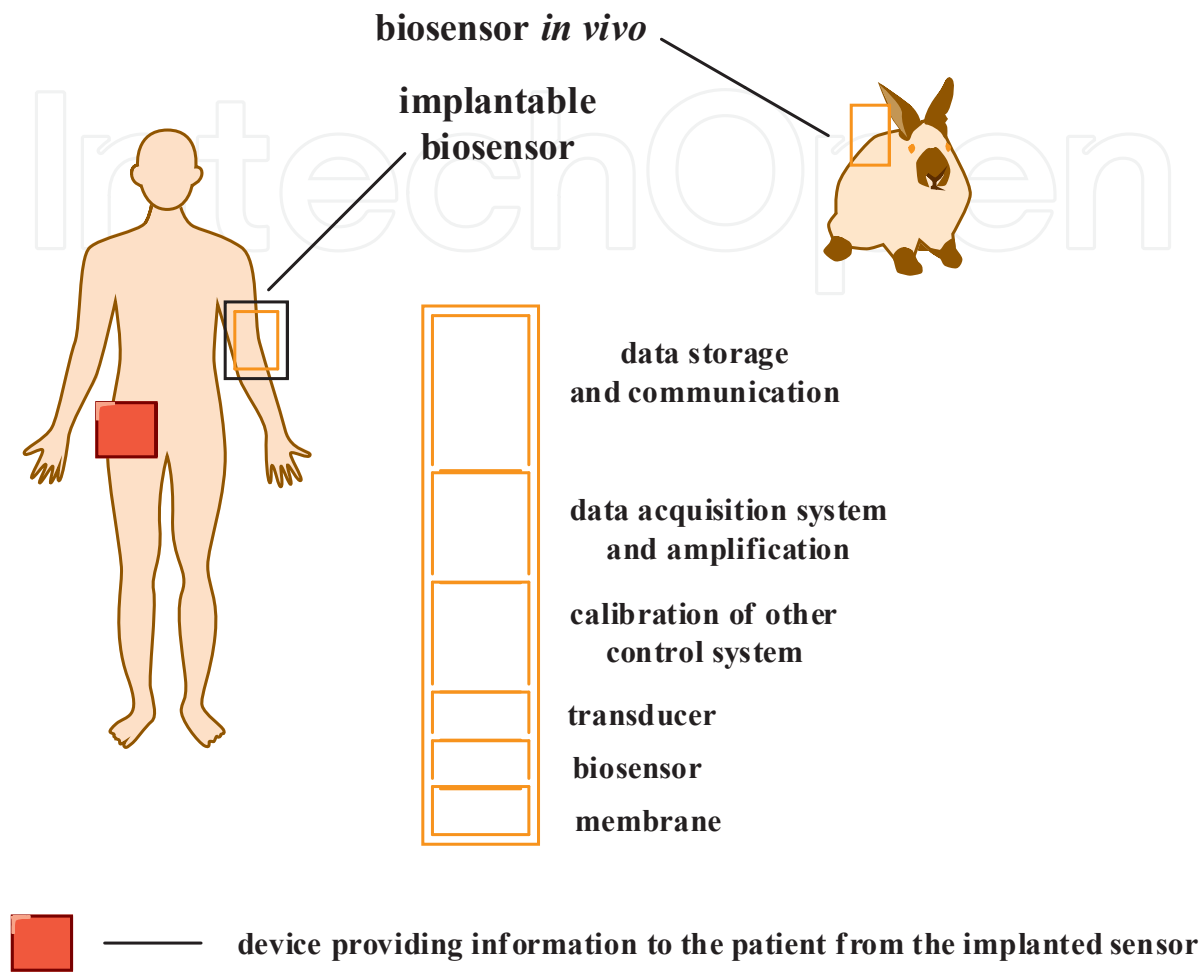


Figure 6. Analytical task of developing *in vivo* biosensor (according to [96])

In vitro sensors have to manage with the same demands in terms. Analytical task of developing and optimizing *in vivo* biosensors of their selectivity, stability is usually not such a critical point. Therefore, *in vitro* sensors also have to be biocompatible in such a way that their presence should not influence the biological medium. Currently, the highly heterogeneous rodent brain is probably most often addressed by *in vivo* and *in vitro* biosensors. Nevertheless, other parts of the rodent central nervous system, the many secretory glands of the regulatory endocrine system, or the tissue of muscles are sites of interest for implantable biosensors [8].

Fixed in a particular brain region for fundamental cognitive, pathological, and pharmacological investigations, the sensing tips of i.e., tapered voltammetric enzyme microbiosensors have demonstrated their ability to directly record up-and down-regulations of neurospecies that may appear in response to premeditated external stimuli such as feeding, drug administration, or gratification at the local level with high time resolution.

Biosensors that are implanted in human body, on the other side, are supposed to control the dynamics of the levels of metabolites related to mental or physical disease states [97]. To achieve this crucial goal they may be placed subcutaneously, just beneath the carrier's skin, or at deeper body locations close to target organs (kidney, liver, and pancreas or the muscles) [97]. Specific applications of biosensors in the human body include clinical point-of-care testing in hospital settings and personal diabetes management. Moreover, the main stream in this task of biosensor development is not on the description of a number of examples from *in vivo* and healthcare measurements but advancements that were reported in the last five years in terms of the design and quality of electrochemical biosensors for successful analysis in a firmly implanted configuration, be it in animals or humans [96]. For detailed information on specific examples of both classical *in vivo* (neurochemical) studies and standard human metabolite monitoring with implanted glucose, glutamate, lactate, acetylcholine, or peroxide biosensors the ones can be referred to several recently published comprehensive articles on the two subjects [98-102]. The clever involvement of new enzymes or adapted enzyme blends in the design of implantable biosensors was used to detect physiologically or pathologically relevant biochemical compounds. The release of the well-known purine ATP as potent extracellular signaling molecule was, for example, demonstrated *in vivo* for the *Xenopus* tadpole spinal cord during motor activity with implanted biosensors that had co-immobilized glycerol kinase, glycerol-3-phosphate oxidase, and phosphocreatine kinase [103]. Miniaturized carbon fiber-based biosensors for *in vivo* measurements of acetylcholine and choline has been prepared by means of a co-immobilization of acetylcholine esterase and choline oxidase [104]. The gliotransmitter serine, well-known for a long time to modulate neurotransmission at the glutamatergic synapse, has been monitored in the rat brain striatal extracellular fluid with implanted biosensors employing mammalian *D*-amino acid oxidase as the indicating biological recognition element [105].

The popular neurotransmitter dopamine is typically measured *in vivo* in particular brain sections with direct fast CV at the solid graphite disc of polished glass–epoxy insulated carbon-fiber microelectrodes [106,107]. In an attempt to improve the selectivity of local dopamine measurements in the complex extracellular matrix of brain fluid, an implantable enzyme-based dopamine microbiosensor has been constructed based on the immobilization of tyrosinase in a thin-film chitosan coating of carbon-fiber disc microelectrodes [108].

As with any classical electrochemical biosensor, an implanted biosensor should also have an exceptional selectivity and sensitivity for the target species, a low detection limit, and a fast response time that is well tailored to the time course of the expected dynamic changes in the concentration of the target analyte in the surrounding tissue. There are, however, important additional properties to look for when the purpose is for stable electrochemical biosensor performance in the complex matrix of the bodies of animals or humans. It is very important for *in vivo* brain biosensor analysis, but also crucial for other situations, to obtain a sensitive acquisition of a strongly localized signal from the molecule in question. Small sensor tip size will of course also be beneficial for placement with minimal (brain) cell and surrounding tissue damage. The exploration of glass-or polymer-insulated needle-type carbon or metal microelectrodes as diminutive precursor structures for biosensors offered

an appropriate solution for this problem and no real innovation in this aspect arose in the period under consideration [96].

The second valid issue for success with biosensors in the chronically implanted configuration is sufficient sensor stability over the extended time of data acquisition throughout a trial. According to a lot of significant behavioral studies but basically in the general implantation case the desired period is days if not weeks of measuring time. The long-term quality of the sensor performance is of course impeded by the gradual loss of proper signal generation caused by the foreign body response and contaminating contact of functional sensor entities on the electrode surface, the immobilization matrix, and the immobilized biological recognition element with protein and lipid contents of the immediate physiological measuring environment [109-113].

Between the issues that can be adverse to long-lasting sensor functioning are (i) the fouling of the immobilization layer in the form of a delamination or loss of porosity which is essential for analyte diffusion, (ii) the degradation or denaturation of the biological recognition element, (iii) the passivation of the electrode surface by nonspecific adsorption of proteins and lipids, and (iv) the slow formation of a barrier for substrate diffusion through an ongoing fibrous encapsulation of the biosensor tip.

Several reports have dealt with the adaptation of redox hydrogels employed for the entrapment of the enzymes used *via* either a mild nondegrading biocompatible environment for the active macromolecule or the creation of hydrogel surfaces that are less prone to the adsorption of contaminating (protein) species. Suggestions include a self-cleaning nanocomposite hydrogel membrane [114], biomimetic hydrogels [115], the involvement of surfactants in the formation procedure of redox hydrogels [116], and hydrogels with optimized type and ratios of individual polymerizing components [117]. According to the fact that nitric oxide effectively inhibits platelet and bacterial surface adhesion, Shin and Schoenfisch proposed advanced biosensor interfaces with a high potential to resist biofouling *via* the implementation of an additional nitric oxide-releasing top coating made of *N*-diazonium diolate-modified polymers [118]. Self assembling polyelectrolyte – poly(ethylene glycol)-based nanofilm multilayers have been demonstrated on porous alumina species as an effective diffusion-controlling and protein adsorption-resistant coatings and were reported as optimized dual-function immobilization matrices for implanted biosensors [119]. Also recommended as surface modifications with promising biocompatibility properties were apparently low-fouling zwitterionic carboxybetaine methacrylate coatings [120], microporous collagen scaffolds that minimized unfavorable tissue reactions while stimulating angiogenesis in the vicinity of biosensor tips [121], new hydrophilic poly(ethylene glycol)-based redox copolymers bearing electrochemically active ferrocene and thiol/disulfide functionalities for anchoring to a gold electrode surface [122], and special nanoporous membranes [123,124].

This time, the effective blood glucose measurement and control is a top of analytical task in medical diagnostics and healthcare, respectively. Already prior to the period covered by this section, direct self-monitoring of internal glucose levels became routine in small-volume blood samples. The required commercial tools and information on both their technology and on the glucose meter marketplace is available, for example, in [100,101,125]. Glucose meters typically

take advantage of sophisticated single-use screen-printed arrays of electrodes one of which is designed as the glucose sensor *via* specific immobilization of mostly GOx as the biological element and the involvement of artificial free-diffusing redox mediators. Upon placement of a droplet of whole blood, the electronics of the glucose measuring device assesses and digitally displays a glucose equivalent in reasonably short time [101]. Even if carried out several times a day, timed glucose monitoring with external sensors activated at user-chosen intervals obviously has the shortcomings that it fails to report irregular up and downs in between assessments and cannot utilize trends associated with daily habits of diabetics for an instant therapeutic action.

Electrochemical enzyme biosensors for *in vivo* studies and human body metabolite monitoring have in recent years been brought to quite an advanced level. A clear proof of the achievements is the good number of successful biosensor recordings of brain activity and the enterprise of marketable continuous blood glucose monitoring. Further improvements in the spatial and time resolution of *in vivo* measurements would need further sensor miniaturization and tapered nanobiosensors that should be similar to their microelectrode analogues in terms of the proper conductor embedment and resistance against sensor fouling. However, they should be equipped with a reduced total tip dimension for better positioning and nanometric sensing areas for fast and highly localized recording.

In vivo biosensor measurements at the single-cell level or at least a very small cell cluster level could then add novel information to the fundamental insights that were and still are gained through *in vitro* single-cell electrochemistry with isolated cells out of their native matrix [126]. Though tapered enzyme-based nanobiosensors with small total tip radii have already been reported [127] but not successfully operated *in vivo*. Here, there is proper space for future innovative research activity. Another area worth working on is the further extension of the lifetime of sensors for continuous blood glucose monitoring and the transfer of the principles of well-working GOx-based implanted biosensors to those incorporating other enzyme systems for broadening the extent of target analytes. The related possible enhancements and expansion of vital health and disease marker monitoring could open up the individualized and portable medication and care plan that is envisioned by clinicians and patients around the world.

6. Summary

At present, scientific researches in the field of biosensing concern not only to the construction of miniaturized devices, faster, more efficient, low-cost, but also to the increase of integration of electronic and biological systems. The future development of biosensors and other bioelectronics of highly sensitive and specific properties demands the combination of interdisciplinary spheres like quantum chemistry and solid state physics as well as surface bioengineering, biology and medicine, electrical engineering, among others. Advances in any of these areas will have significant effect on the future of medical and environmental diagnostics where invention of more effective, real time monitoring procedures will be advantaged by biosensing technologies.

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