Electrochemical parameters and techniques in drug development, with an emphasis on quinones and related compounds

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This review article summarizes recent applications of electrochemical techniques to redox-active drug development and mechanistic studies. It includes a general introduction to the use of electrochemistry in biology, with a focus on how electrochemistry can uniquely provide both kinetic and thermodynamic information. A number of studies are reported from the literature and the authors' laboratories, including the investigation of reactive oxygen species, biooxidative/bioreductive activation of pro-drugs, and DNA alkylation, with a particular emphasis on quinones and related compounds. Data from techniques ranging from traditional cyclic voltammetry to sophisticated single cell studies are presented. The examples herein presented illustrate how electrochemical, biochemical and medical knowledge can be integrated to develop strategies for the design and development of redox-selective therapeutics.

1 Introduction

The subject of this review is the use of electrochemistry in developing and understanding the chemical and biochemical mechanisms of potential drugs which are activated by and/or influence the redox environment of the target cell. The rational design of drugs that interact with redox machinery of the cell is a new field, although mechanisms of action involving electron transfer and reactive oxygen species (ROS) generation have been recognized even for well known drugs with long clinical use.[1] An important advantage to this drug design strategy is related to potential selectivity in killing diseased cells. For example, a compound which is activated by either oxidation or reduction can exploit the particular redox status of a diseased cell, while remaining inactive in healthy cells. This idea has been particularly developed in the design of hypoxia-selective drugs and imaging agents. Another tactic is to create molecules that disrupt cellular homeostasis, for example, by producing reactive oxygen or nitrogen species (ROS, RNS). This is especially interesting in cases, such as some cancers, where cells are under oxidative stress. The release of additional RONS could overwhelm the regulating machinery of the cell or induce apoptosis. Our focus will be on how one can take advantage of perturbations of the redox environment within cells to create novel therapeutics.

The redox environment of a living cell essentially refers to its oxidizing or reducing capacity. In the most general sense, it can be related to the state of the many redox couples present in the cell, some of which are directly coupled and interacting *via* enzymes, and has been variously defined in terms of the status

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of the NAD^+/NADH, $NADP^+$/NADPH,[2] or GSSH/GSH couples.[3] The redox status of a given biological environment is crucial, because numerous fundamental and deleterious processes in living cells are governed and stimulated by redox reactions through many feedback loops which may eventually trigger shock proteins. For example, cellular respiration, the conversion of biological energy into the useful ATP form, occurs by the cell's mitochondrial respiratory chain, which involves a series of reactions including the oxidation of sugars and the reduction of NAD^+ and oxygen. Redox enzymes, which catalyze reduction and oxidation reactions, are ubiquitous. Proper performance of this delicate machinery necessitates a finely tuned redox environment, which is maintained by nutrients, peptides, proteins, and gene expression;[4–6] any change to this tuned environment can alter the biological homeostasis.[3,7]

Electrochemistry is the standard method for studying redox reactions, and the literature is replete with electrochemical techniques as applied to biology. The bio-electrochemical literature can be generally divided into the following categories: (1) advanced instrumentation such as scanning electrochemical microscopy[8–10] and electrochemical impedance spectroscopy;[11] (2) the study of redox enzymes, often as thin films on electrodes;[12–14] (3) the use of electrochemistry as an analytical tool for the quantification of biologically active molecules in a sample;[15] (4) electrochemical biosensors;[16–18] (5) the study of drug reaction mechanisms and their correlation with biological activity;[19] and (6) redox active drug development; the last two topics being the main focus of this review. Over the years, these two important areas have received less attention than is deserved, since they address the very important but difficult aspects of energetics and kinetics of electron transfer.[19]

2 Practical considerations

2.1 Mimicking biological conditions

For obvious reasons, researchers studying drug mechanisms attempt to model cellular conditions as closely as possible, even to the extent of using *in vitro* cell cultures for which specialized electrochemical experiments have been developed. The importance of mimicking biological conditions in non-cellular media is a non-trivial problem due to the great diversity found in the cellular and extracellular environments. For example, all cells contain both hydrophilic regions (*e.g.* cytoplasm) and lipophilic regions (*e.g.* endoplasmic reticulum, membranes, enzyme active sites). Similarly, compartments of a cell often possess different regulating systems and thus different redox environments. These have been classified in the following order from more reducing to more oxidizing: mitochondria > nuclei > cytoplasm > endoplasmic reticulum > extracellular space.[6] Additionally, the redox environment alters during the normal life cycle of the cells, from more reducing (proliferation, differentiation) to more oxidizing (apoptosis).[20,21] Another important factor is related to the O_2 content of the cell and its participation in side-metabolic routes, which strongly influence the outcome of biological redox reactions.[22] Some tissues, for example solid tumours, contain regions of low oxygen tension (hypoxia), generally thought to arise as a consequence of a poor and disorganized blood supply.[5] The pH can also differ within tissues; *e.g.* breast cancer cells have been shown to acidify the extracellular space, compared to normal breast cells.[23] All these facts must be considered in the attempt to mimic the role of biological environments.

Fortunately, the versatility of electrochemical methodology allows the modelling of a multitude of biological milieus. Different ranges of pH and oxygen content in the electrochemical cell and solvents with diverse physicochemical and chemical properties can be used. However, systematization is urgently required, in terms of methods, electrodes, supporting electrolytes, *etc.*, to allow a more general use of the huge bulk of already available data.[24]

The usual parameters normally obtained and employed, especially in cyclic voltammetry, the method most used, are the potentials of the oxidation (E_{pa}) and reduction (E_{pc}) peaks or E_{redox} $(E_{pc} + E_{pa})/2$ (for reversible systems) or $E_{pc} - E_{pc/2}$ (for irreversible ones), the magnitude of the current function $I_p/(v^{1/2}C)$ and the ratio between the anodic and cathodic currents I_{pa}/I_{pc}. The potential E_{redox} or similar parameters, $E_{1/2}$, in polarography, give a quantitative measure of the ease of reduction of an oxidant or electron acceptor, A, since the more positive the value of the potential of the couple $E(A/A^{\bullet -})$, the more powerful the oxidant. Similarly, the more negative the value of $E(A^{\bullet -}/A^{2-})$, the more powerful the reductant.[24,25] Additional information can be obtained from books[25] aimed to non-electrochemical readers.

2.2 Electrochemistry caveats

The difficulty in comparing data[24] is due to the fact that electrochemical mechanisms depend strongly on experimental conditions, especially in non-isotropic environments. Several complications are evident. Firstly, the reduction potential by a single electron to form a radical or radical anion is generally quite different from reductions which generally involve one or more pairs of electron transfers intertwined with classical chemical reactions (*e.g.*, acid–base, nucleophilic, electrophilic, bond cleavage reactions, *etc.*); the same can be said for oxidation profiles.[24] In addition, electrochemically generated intermediates are generally radicals, and easily undergo a panel of chemical reactions, such as disproportionation, dimerisation, rearrangements, *etc.*, which are seldom encountered in non-paramagnetic chemistry.

Of great importance are molecules and intermediates with ionisable groups or sites for protonation, which show pH-dependence, and may often cause problems and affect correlations.[24] Potentials shift greatly in water compared to less protic or aprotic media; reduction potentials in aprotic media are normally more negative (reduction) or more positive (oxidation) than those measured in protic media, because protic media allow proton transfer to and from the electrogenerated anion or cation intermediates, leading to their fast stabilization.

All these features may make the electrochemical data not directly comparable with the classical thermodynamic data which are ordinarily sought from electrochemistry. Indeed, the

above kinetic features often result in a redox wave potential different from that predicted by thermodynamic parameters, (*e.g.*, the Nernst's true or apparent potential of a redox couple) unless the whole process is so fast both ways that it respects thermodynamics. Though electrochemical data should then be manipulated with caution since they reflect both thermodynamics and kinetics, it must be noted that the same situation applies to the cellular environment. Indeed, most redox active centres belong to proteins or enzymatic pools which are anchored into membranes. Therefore what may appear as a specific feature to molecular electrochemistry may in fact also apply to a cell, owing to the spatial constraints which affect the transport to and from active redox centres, similar to the process which occurs in electrochemistry. Conversely, the usual Nernst standard or apparent potential relates to homogeneous isotropic conditions which are seldom applicable to cells.

However, a further problem is that, in the literature, potentials may often be quoted relative to different reference electrodes (NHE, calomel or Ag|AgCl) without clearly specifying either the conditions used or the reference, which is often implicit for authors. A standard reference compound is useful in making comparisons between these potential scales. For example in organometallic electrochemistry where this problem often arises, ferrocene is generally used as such an internal reference, although the ferrocene/ferrocenium couple is not entirely immune to variations in its environment.[25] However, shifts in potential in a series of structurally related compounds measured in the same solvent against one standard will often be linearly related to the behaviour in another solvent, though with a ρ^{Hammet} slope which may differ from unity.[26]

The differences between reversible and irreversible electron transfers can also play an important role. In some cases, the standard potential for reactions involving slow heterogeneous electron transfer, *e.g.* dissociative electron transfer, is not easily determined using simple electrochemical methods since the direct reaction is subject to a large overpotential in order to compensate for its slow kinetics. As a result, reduction/oxidation potentials measured from cyclic voltammetry are not, themselves, an accurate indication of the standard potential and cannot be compared directly to the oxidation/reduction potential of chemical or biological partners to decide whether a particular electron transfer will be feasible under physiological conditions, though correlations may exist.[26] In those cases, thermochemical cycles or correlations are often used to estimate the standard reduction/oxidation potential.[27,28] An additional series of difficulties, truly specific to electrochemistry and not met with in biological situations, is related to adsorption of the analyte onto the electroactive surface or to speciation effects. Both are difficult to control, and have negative effects on reproducibility, though they may not be easily avoided. Several techniques are available to decrease the problem of adsorption, but they may not be practical under all biological conditions.[25]

3 Principal themes in the electrochemical study of drug mechanisms

Despite the above intrinsic difficulties, electrochemical techniques have been extensively used to clarify drugs' mechanism of action, providing excellent insights into their mode of activity, and inspiring further drug design. The main contributions, which are in the field of cancer research, combine electrochemical and spectroscopic methods, in particular those used to analyze free radicals, (*e.g.* ESR).†[32–34] These studies mainly focus on the activation of drugs by reduction or oxidation, and/or their influence on redox homeostasis.

3.1 Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (RONS), including the primary unstable species $O_2^{\bullet -}$, $NO^{\bullet}$, their direct products H_2O_2, peroxynitrite (ONO_2^-), and $OH^{\bullet}$, as well as their follow-up products, are involved in normal cellular metabolism in mitochondria and peroxysomes, and are produced from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS or RNS production, such as environmental toxins, *etc*. A sophisticated enzymatic and non-enzymatic antioxidant defense system, including catalase, superoxide dismutase, glutathione peroxidase, glutathione, tocopherols and vitamins, counteracts and regulates overall ROS levels to maintain physiological homeostasis through chemical destruction (disproportionation) or diversion towards less harmful radicals or diamagnetic species.

An overabundance of RONS is generally referred to as the condition of ''oxidative stress''. Although some researchers have defined oxidative stress as simply a global imbalance of pro-oxidants and antioxidants, this view seems to be inadequate and conceptually limiting. The accumulated data show that a more suitable definition for oxidative stress is a condition that disrupts redox signalling and control.[6,20,35] Regardless of how or where ROS, RNS, RSS, RCS and RClS are generated, a rise in intracellular oxidant levels has two potentially important effects: triggering of the activation of specific signalling pathways (Fig. 1) and damage to various cell components (Fig. 2).[36] Notably, the spontaneous and controlled ''oxidative burst'' of RONS by neutrophiles or macrophages during phagocytosis is a major non-specific mechanism of host defence against bacteria and dead cells.[37–39] More insidiously, oxidative stress has been connected with protein[40] and lipid oxidation,[41] DNA mutagenesis,[42] ageing,[20,35,36,43–45] and diseases[35] such as asthma,[46] cancer,[47,48] atherosclerosis,[49] Alzheimer's disease,[50,51] diabetes,[52] and rheumatoid arthritis.[53] Indeed, there is a nutritional industry estimated to be worth over a half a billion dollars which exists to supply customers with antioxidant agents to theoretically ward off oxidative stress,[54] and the literature abounds with a variety of antioxidant assays applied to foods and chemicals, though the

† Pulse radiolysis has also been intensively used and gives fundamental contributions to the area. It allows the measurement of monoelectronic reduction potentials in aqueous solutions (E_7),[29,30] which is difficult to obtain through electrochemical methods due to the fast reaction of radical intermediates. This is minimized in aprotic media by stabilization of these intermediates and correlations have been reported to hold within a series of chemically related substrates.[31] However, there are no such reports regarding quinones because of the complexity of the square-scheme mechanisms in which these species are involved, and the drastic effects of simple H-bonding on their electrochemistry. Though this would be extremely useful for biological mechanism rationale, and despite a similar focus on electron transfer, this technique is beyond the scope of this review.

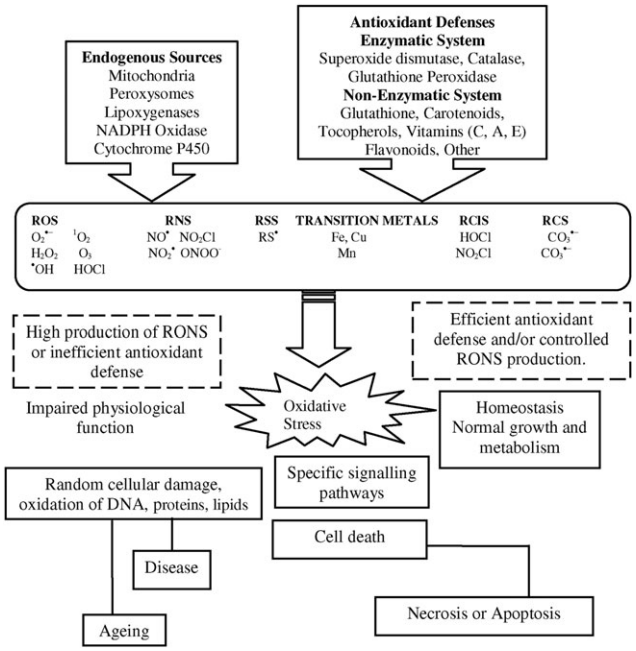

Fig. 1 The sources and cellular responses to reactive oxygen, nitrogen, sulfur, chlorine, and carbon species and transition metals (M^n). Adapted with permission from ref. 36. Copyright 2000, Nature, Macmillan Publishers Ltd.

situation is not well understood even for the widely used diet supplement Vitamin C.[55]

3.2 Oxidative stress and drugs

The electron transfer-reactive oxygen species-oxidative stress theory (ET-ROS-OS), advocated by Peter Kovacic, represents a broad and unifying background rationale of drug-action that can aid in drug design.[1,19,57] This approach is particularly appropriate to disease states which are associated with oxidative stress in the diseased cells.[5,58–60] There is increasing evidence of ET-ROS-OS involvement in the mechanism of action of a wide variety of physiologically active compounds,[57,61] such as quinones,[62] nitroaromatics[63] and imminum salts.[57,64] These electroactive compounds are represented as A in Fig. 3, being denoted globally as redox cyclers.[57]

The development of ROS-enhancing drugs is currently an active field of cancer research.[65,66] It has been shown that some cancer tissues are in a state of oxidative stress, and their antioxidant machinery is working at full capacity. It has been

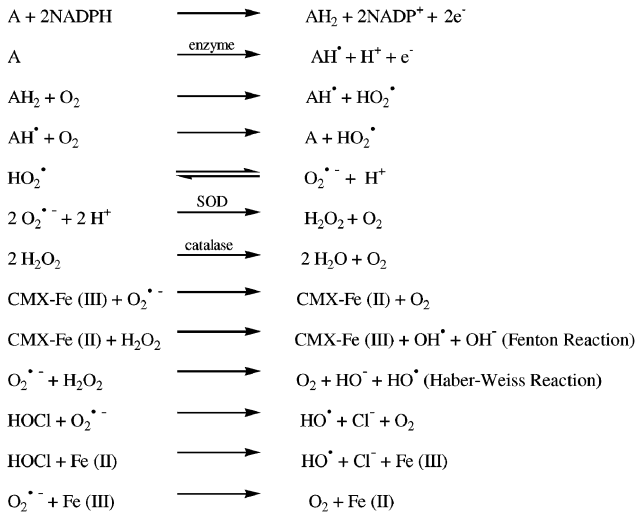

Fig. 3 Pathways related to oxidative stress. SOD = superoxide dismutase; CMX–Fe(III) = iron complexed with protein or ATP. A: electroactive substances (reversible electron transfer, redox cyclers).

postulated that additional ROS will overwhelm these cells but not normal cells, and thus ROS generating compounds may show selectivity for some types of cancer.[58,67] It should also be mentioned that elevated levels of ROS are also known to trigger apoptosis, or programmed cell death.[68] Currently explored strategies to enhance oxidative stress intensity or effects include agents that directly increase ROS in cells to lethal levels; agents that inhibit antioxidant enzymes;[69] agents that tilt the intracellular redox balance to more oxidizing potentials; and catalysts that enhance the toxicity of ROS.[5]

3.3 Bioreductive/biooxidative formation of active species

Redox activation of otherwise inactive pro-drugs, often followed by further chemical transformations, such as hydrolysis, can generate highly electrophilic compounds that react with endobiotics. A successful bioreductive/biooxidative prodrug possesses the following properties: minimum toxicity to healthy cells; stability toward metabolism in aerobic cells, and suitable pharmacological and solubility properties.

In thermodynamic terms, if the reduction potential of an electroactive compound in buffered aqueous media (pH = 7) is more positive than −0.5 V *vs.* NHE, the enzymatic transfer of electrons is possible *in vivo*. A large number of physiologically active compounds, such as quinones and nitroarenes,

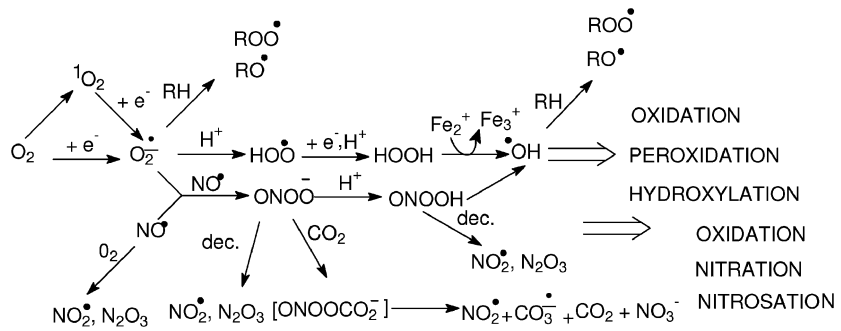

Fig. 2 Generation and fates of ROS and RNS. Adapted from ref. 56.

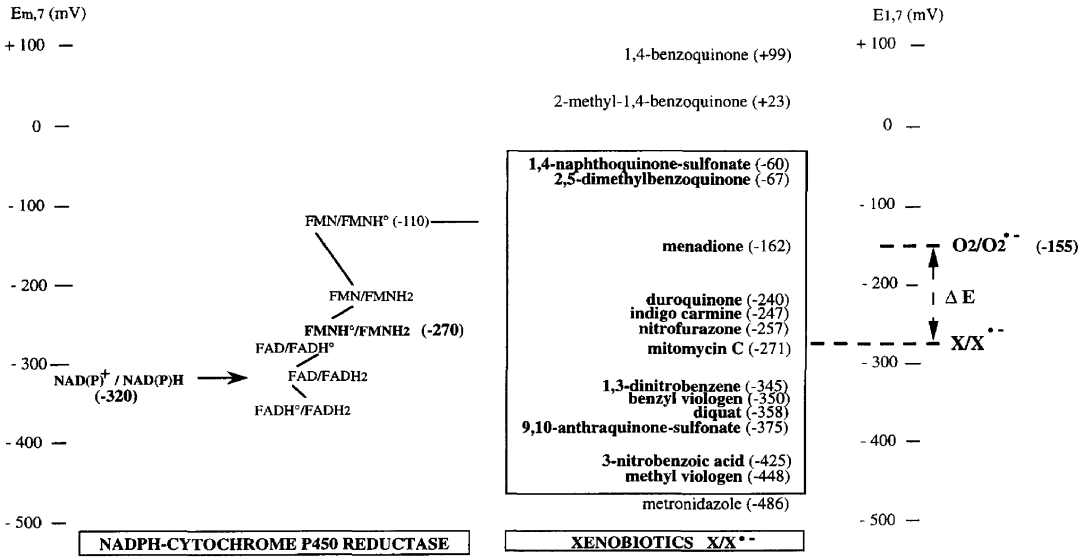

Fig. 4 Redox potentials (pH 7.0) of reductases: NADPH-cytochrome P-450 (FAD-FMN, E_m, 7) and monoelectronic reduction potentials for xenobiotics (E^1_7). The values for enzymes were determined by Iyanagi *et al.*[71]. Reprinted with permission from M.-H. Livertoux, P. Lagrange and A. Minn, The superoxide production mediated by the redox cycling of xenobiotics in rat brain microsomes is dependent on their reduction potential, *Brain Res.*, 1996, **725**, 207–216. Copyright 1996, Elsevier.

display reduction potentials in the range of −0.5 V to 0.0 *vs.* NHE.[57] This range overlaps those of biological reductants (around −0.4 V *vs.* NHE) and that of $O_2/O_2^{\bullet -}$, *ca.* −0.2 V *vs.* NHE (Fig. 4). This range could be slightly or greatly extended by concentration effects or by fast consumption of products, respectively, since these latter induce fast displacement of redox equilibria (Le Chatelier principle). Fig. 4 allows the comparison between redox potentials of enzymatic systems (pH 7, *vs.* NHE) and reduction potentials (E^1_7) of xenobiotics.[70]

The one-electron reduction standard potential of oxygen is −0.18 V (but only when $[O_2] = 1$ M which does not hold in aerobic biological conditions) and −0.65 V (when $pO_2 = 0.2$ atm) *vs.* NHE. The former value is more appropriate for comparison with the reduction potentials of drugs expressed in the same way but is not appropriate under real physiological conditions owing to the scarcity of O_2. Thus, taking into account both the minimum reduction potential necessary for activation by common flavoproteins, and the maximum potential for protection against oxidation by oxygen, the one-electron reduction potential required for most types of hypoxia-selective, bioreductive drugs is in the region of −0.5 to −0.1 V (*vs.* NHE, in water, pH 7) though this may be extended positively when the target electron-transfer initiates a downhill thermodynamic cascade of events.

The relative one-electron reduction potentials of drugs control the position of the equilibrium defining the interaction of drug anion radicals with molecular oxygen (eqn (1)).[29]

$$\mathrm{Drug}^{\bullet -} + \mathrm{O_2} \overset{K_1}{\rightleftharpoons} \mathrm{Drug} + \mathrm{O_2}^{\bullet -} \qquad (1)$$

The equilibrium constant K_1 is approximately equal to $10^{\Delta E/0.06}$, where $\Delta E = [E(O_2[1\ \mathrm{M}]/O_2^{\bullet -}) - E(\mathrm{drug/drug}^{\bullet -})]$ and the potentials are expressed in volts. Thus to a first approximation $K_1 > 1$ if E (drug/drug$^{\bullet -}$) < -0.2 V *vs.* NHE. Conversely, the forward reaction is increasingly disfavoured at more positive potentials, although it will be enhanced if superoxide dismutase activity or a specific reagent prone to react with the superoxide is present, since the effect is to continuously displace the equilibrium in eqn (1) by removing $O_2^{\bullet -}$ rapidly from the area.[29]

Similar studies were performed in aprotic media, to mimic membrane-bound NADPH-Cytochrome P-450 reductase and other lipophilic enzyme active centres. The reduction potentials, thus obtained, are more negative than in aqueous media; therefore the values calculated for aqueous media, in terms of *in vivo* reduction, are now $E_{redox} = -0.70$ to −1.10 V *vs.* SCE.[70]

4 Selected applications of electrochemistry in non-cellular media

Redox active therapeutics, including chemopreventive antioxidant treatments, is a vast and rapidly expanding area of research.[58,59] Most of the present views of research in the field of oxidative stress (OS) and cancer date back less than 10 years. Intricate aspects of ROS up-regulation, antioxidant defence and resistance toward OS are just becoming apparent; for example, in oxidatively stressed cancer cells, the excess superoxide and peroxide production may promote cancer cell proliferation, though it only damages normal cells. Indeed, the fact that the redox make-up of the cancer cell can be distinctively different from that of healthy cells allows the design of selective redox-activated agents, which do not need a complicated drug-delivery system. Some tumours, such as solid lung carcinoma, are hypoxic, and its cells are therefore more reducing than normal, while others, such as those of breast and prostate cancer, are under increased oxidative stress compared to normal cells.[5,21,58,59] These approaches are also rewarding in the field of tropical diseases, as shown in recent reviews.[60,72–74] Such recognition is essential when looking for

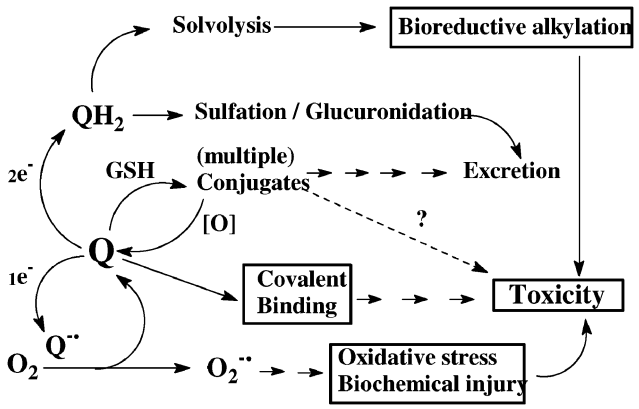

Fig. 5 Biological fates of quinones (Q). Reprinted with permission from T. J. Monks, R. P. Hanzlik, G. M. Cohen, D. Ross and D. G. Graham, Contemporary issues in toxicology: Quinone chemistry and toxicity, *Toxicol. Appl. Pharmacol.*, 1992, **112**, 2–16, Copyright 1992, Elsevier.

drugs susceptible to interference with the mechanisms of a target cell. In other words, one species may act diversely, with positive or negative effects on a cell's longevity, depending on the cell status. This is evidently the case for quinones, which are reputed to be antioxidants in normal cells, (*viz*., the famous French paradox associated with the moderate consumption of quinone-rich wines) but induce toxic pathways in abnormal cells.

4.1 General mechanisms of quinone cytotoxicity

Quinones have been employed extensively as models to study cellular mechanisms of chemical induced toxicity. They are oxidants and electrophiles, but, because nucleophilic addition to a quinone represents a formal two-electron reduction, these properties are inter-related. Indeed, the striking feature of quinone chemistry is the ease of reduction and therefore the ability to act as oxidizing or dehydrogenating agents, the driving force being the formation of a fully aromatic system.[24,75] Two major mechanisms of quinone cytotoxicity have been proposed: stimulation of oxidative stress and alkylation of cellular nucleophiles, which encompass a large range of biomolecules.[76–79] Quinones play a major role as bioreductive drugs, OS enhancers, and redox catalysts (Fig. 5).

The toxicology of quinones is modulated by the presence of substituents that effectively determine the relative participation of their oxidant and electrophilic properties. For instance, the presence of an electron-withdrawing group confers stronger oxidant properties on the quinone, but the corresponding hydroquinone or catechol is less readily oxidised. Conversely, with electron-donating substitution, the oxidant power is less pronounced, but the corresponding hydroquinone or catechol is more easily oxidised.

The mechanism of action of quinoid antitumour agents have been thoroughly investigated (Fig. 5). Under aerobic conditions, *i.e*. in tumours with sufficient blood supply, a one-electron reduction predominates, resulting in free-radical intermediates which can react with molecular oxygen (eqn (1), Fig. 3) and ensuing superoxide production. This can cause additional damage to the DNA of the tumour cell, but, frequently, this is non-specific and may also induce unwanted damage to normal cells, leading to serious side effects. An alternative pathway of activation involves a two-electron reduction of the quinone function, which may then be inactivated by subsequent glucuronidation and/or sulfation, or by the conversion of the hydroquinone into an alkylating intermediate, the quinone methide.[59] Such a pattern is believed to predominate under anaerobic conditions or inside anaerobic loci within a cell.

The electrochemical properties of quinone compounds are obviously very important for their bioreductive activation, either to the semiquinone or to the hydroquinone. There are several examples of correlations between electrochemical potentials and biological activities. For example, a definite correlation has been found between redox potentials and the inhibitory effects of naphthoquinones on Epstein–Barr virus early antigen activation[80] and with their cytotoxicity.[81] There are also several examples of quinone activity outside the cancer area. Chagas' disease is a long term debilitating disease caused by the flagellate protozoan *Trypanosoma cruzi*, transmitted by triatomine insects and by blood transfusion. It is one of the most serious endemic parasitic diseases of Latin America, with a social and economic impact far outweighing the combined effects of other parasitic diseases.[82] A special feature of *T. cruzi* is its unique sensitivity to the action of intracellular generators of H_2O_2. *T. cruzi* possesses an original redox defence system, based upon trypanothione and trypanothione reductase, a NADPH-dependent flavoprotein, which regenerates trypanothione from its oxidised form (disulfide form). It lacks catalase and glutathione peroxidase, being therefore substantially more sensitive to H_2O_2-induced oxidative stress than its biological hosts. To date, Chagas' disease has defied all attempts to develop an efficient chemotherapy. Despite the recognition of the importance of redox cyclers as potent trypanocidal agents, few reports have shown a possible correlation between redox potentials and trypanocidal activities. Several naphthoquinones were assayed as trypanocidal and their E_{redox} were measured in aprotic medium, using Hg as the working electrode.[83] These results suggested that it is more likely to observe trypanocidal activity among those quinones displaying their first reduction wave at potentials more positive than -0.72 V *vs*. SCE, especially if they are *ortho*-naphthoquinones.[83] It is easier to find positive correlations between redox potentials and biological activities when the mechanism of action is ET-ROS-OS, explaining the importance of studies in the presence of oxygen.

4.2 Electrochemical studies of quinones in the presence of oxygen

Cyclic voltammetry investigations of quinones in the presence of oxygen in aprotic media have been considered as a useful tool for studying the interaction of oxygen and the superoxide anion radical with quinones and their radical anions.[84] Non-aqueous aprotic solvents should provide better models of membrane environment in which peroxidation processes take place, because both the superoxide anion radical and its conjugated acid, the hydroperoxyl radical, are unstable in water and other protic solvents, owing to their fast

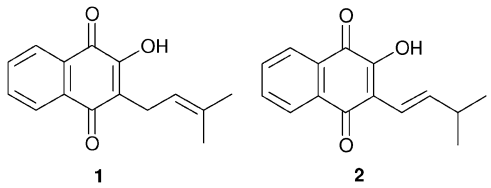

Fig. 6 Lapachol (**1**) and isolapachol (**2**).

disproportionation.[84] For peroxidation in the presence of oxygen to occur, the presence of hydroxyl groups in the quinone moiety has been demonstrated to be essential. It was also shown that the rate constants of the electron-transfer reactions from semiquinone anion radical to molecular oxygen increase with decreasing pK_a of the hydroxyquinones.[84] This stems from the fact that the corresponding phenolate ion is more easily oxidised than the phenol parent. Thus, in terms of cytotoxicity and pharmacological activity, the presence of hydroxyl groups and their acidities are of fundamental importance.

4.2.1 Reduction of lapachol and isolapachol and their interaction with oxygen. As examples, electrochemical studies of lapachol (NQOH, **1**) and isolapachol (ISOH, **2**) were performed and are included as an illustration of the concept (Fig. 6). Lapachol (**1**) possesses antitumour, antibiotic, antimalarial, anti-inflammatory and antiulceric activities.[85] Recent results showed that **1** and **2** have significant activity against several etiological agents of tropical diseases, including *Trypanosoma cruzi*, *Leishmania braziliensis*, *L. amazonensis*, and also, indirectly, killing the mollusc *Biomphalaria glabrata* (adult snail and egg masses), the intermediate host of *Schistosoma mansoni*, the causative agent of schistosomiasis.[83,86–89]

Cyclic voltammetry studies, in aprotic media (DMF + 0.1 M Bu_4NClO_4 or DMSO + 0.1 M Et_4NPF_6), on glassy carbon and/or platinum electrodes were performed with **1** and **2**, in the absence and presence of oxygen, in order to investigate their electrochemical reduction mechanism and possible oxygen interaction with the electrochemically generated radical anion (Fig. 7). The electrochemical behaviour is complex,[90] but reproducible in relation to 2-hydroxynaphthoquinones.[91] The first reduction peak (Ic) is related to semiquinone formation, but is complicated by the occurrence of self-protonation mechanisms and the formation of hydrogen-bonded intermediates.[90,91] The observed anodic shift in the potential of the first reduction wave of isolapachol, in comparison to lapachol, is related to the higher acidity of the enolic group.

Addition of O_2 to the system causes remarkable changes to the position of the first reduction peak potential (E_{pIc}, eqn (2)) as well as to the shape of the curves[90] of **1** and **2** (Fig. 7).[92] These similar effects include (a) the increase of the height and anodic shift of the first cathodic wave Ic, related to the generation of the semiquinone, (b) disappearance of the corresponding anodic wave Ia and shoulders IIc, and (c) increase of the wave (IIIc) related to the reduction of the conjugated base of **1** and **2**. The oxygen reduction wave potential was also affected by the presence of the quinones, undergoing a positive shift driven by protonation of the superoxide ion by ISOH, or because of an overall H-atom transfer from $ISOH^{\bullet -}$ to O_2 (eqn (3)).

$$ISOH + e^- \rightleftharpoons ISOH^{\bullet -} \quad (2)$$

$$ISOH^{\bullet -} + O_2 \rightarrow ISO^- + HOO^{\bullet} \quad (3)$$

The production of superoxide species in DMSO solutions upon interaction of anion radicals of the hydroxy derivatives of anthraquinones was demonstrated by UV-spectroscopy[84] and the favourable energetic effect of the overall sequential electron and proton transfer from hydroxynaphtoquinone

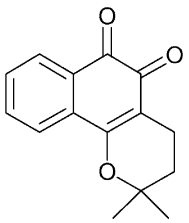

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Fig. 8 β-Lapachone (**3**).

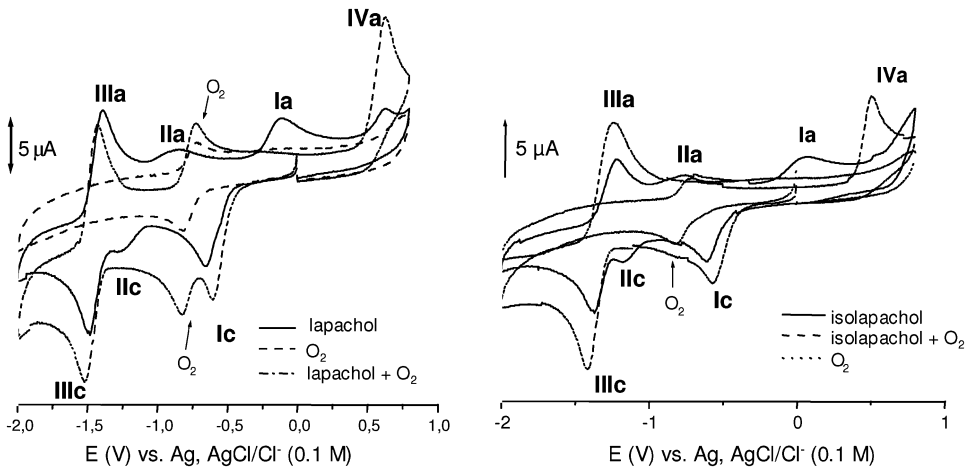

Fig. 7 Cyclic voltammetry of **1** and **2** in the presence or absence of O_2, glassy carbon electrode, DMSO + 0.1 M Bu_4NClO_4; $c1 = c2 = 1$ mM, $\nu = 0.100$ V s^{-1}.

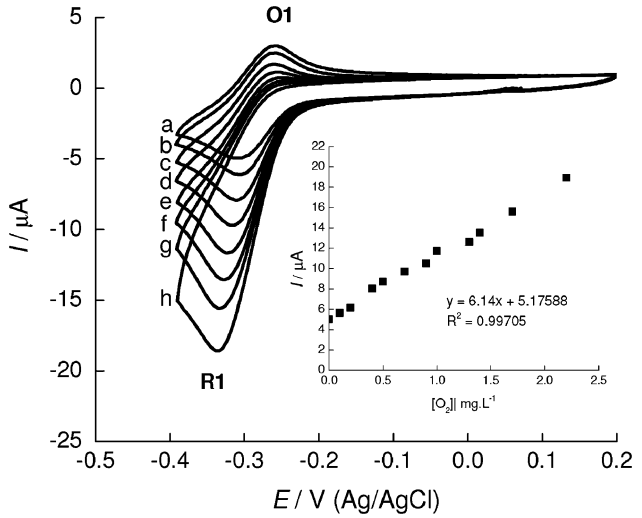

Fig. 9 Cyclic voltammograms of 0.10 mM of **3** in PBS buffer (pH 7.4 + 10% EtOH), on glassy carbon electrode (3 mm diameter), in the presence of different oxygen bulk concentrations: (a) 0, (b) 6.25, (c) 12.5, (d) 21.87, (e) 31.25, (f) 43.75, (g) 53.12, and (h) 68.75 μM, $\nu = 50$ mV s^{-1}; Inset: plot of $I_{p_{R1}}$ as a function of oxygen concentration. Reprinted with permission from F. C. de Abreu, D. C. M. Ferriera, M. O. F. Goulart, O. Buriez and C. Amatore, Electrochemical activation of β-lapachone in β-cyclodextrin inclusion complexes and reactivity of its reduced form towards oxygen in aqueous solutions, *J. Electroanal. Chem.*, 2007, **608**, 125–132, Copyright 2007, Elsevier.

anion radical to molecular oxygen was demonstrated by high-level *ab initio* calculations.[93,94]

4.2.2 Reactivity of the reduced form of β-lapachone towards oxygen. Among the naturally occuring cytotoxic naphthoquinones, β-lapachone (**3**, Fig. 8) has been the target of many investigations during recent years. This quinone can be isolated from plant extracts of *Tabebuia avellanedae*.[85] It has been intensely investigated for clinical use as a trypanocidal agent[83] and against HIV-1 replication, showing suppression in both acute and chronic infection. One of the most important applications of this compound is its action against cancer.[95–99]

Based on the facts already described about quinones and from the experimental biochemical evidence of the reactivity of the radical anion with oxygen (eqn (1)) for this quinone,[100] the reactivity towards oxygen of the reduced form of **3** was studied by cyclic voltammetry. The cyclic voltammogram of **3** obtained in a mixed ethanolic (10%) aqueous buffered media (PBS, pH = 7.4) exhibited a reversible reduction wave (Fig. 9, curve a).[101] In the presence of oxygen, the peak current of the reduction wave increased in direct proportion to the added oxygen. The wave became irreversible indicating an efficient catalytic process.[102] This behaviour suggests a reaction between the reduced form of the quinone and oxygen that regenerates the starting substrate (eqn (4)), with the likely formation of hydrogen peroxide which is unstable in aqueous media. This electrochemical observation is consistent with the results obtained in the presence of NAD(P)H–quinone oxidoreductase 1 (NQO1)[103] or using biochemical methods.[100] In the former case, it was proposed that NQO1 reduces β-lapachone to an unstable hydroquinone that rapidly undergoes a two-step oxidation back to the parent molecule, leading to a redox cycle.

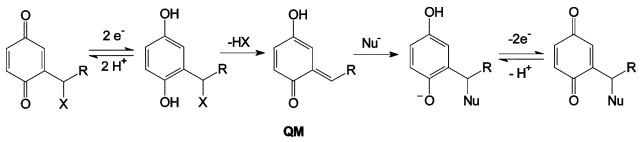

Fig. 10 QM formed by HX elimination from the hydroquinone and nucleophilic attack in a Michael addition type reaction. Adapted with permission from ref. 110. Copyright 2002, American Chemical Society.

$$QH_2 + O_2 \leftrightharpoons [QH_2^{\bullet+}, O_2^{\bullet-}] \rightarrow QH^{\bullet} + HO_2^{\bullet} \rightarrow Q + H_2O_2 \quad (4)$$

An interesting example concerning the reactivity of mono-(arylimino) derivatives of **3** is the hydrolysis of less toxic quinoneimines to release quinones, whose kinetics could be investigated by electrochemical methods.[104] Electrochemistry has also been used to investigate the fate of halogenated biologically active quinones.[105]

4.3 Reductive activation of quinones and DNA alkylation

The vast majority of clinically employed alkylating agents behave as electrophilic traps for molecular nucleophiles. Such nucleophiles often include amino acids and the nucleobases of DNA and RNA.[106] The interaction of drugs with DNA is among the most important aspects of biological studies in drug discovery and pharmaceutical development processes.[107] Prodrugs are normally employed and activation occurs through *in situ* reduction, by such endogenous reductases as cytochrome c, cytochrome b5 and xanthine oxidase,[29,106] in a process which is referred to as bioreductive alkylation. Quinones commonly function as the reducible moiety of these agents due to their facile *in vivo* and *in vitro* reduction, which is followed by different decay mechanisms, including the formation of quinone methide by loss of an anionic leaving group.[105] Determining the role of these intermediates is key to the design and development of effective bioreductive alkylating drugs, and more extensively in the understanding of several biological and toxicological *in vivo* events.[29,106]

Ortho-quinonemethides (*o*-QM) and iminemethides are among the most important activated intermediates.[108–110] The reductive activation of quinone to its *o*-quinone methide, followed by a nucleophilic attack is shown in Fig. 10.

Many compounds bind and interact with DNA causing changes in structure and/or base sequence. Moreover, ligand binding at a specific site on the DNA can induce long range effects on both DNA structure and stability.[111] Intercalation and groove-binding are the two most common modes by which small molecules bind directly and selectively to DNA.[111] Intercalation, which is an enthalpically driven process, results from the insertion of a planar aromatic ring system between DNA base pairs with concomitant unwinding and lengthening of the DNA helix.[112] In contrast, groove-binding, which is predominantly entropically driven, involves covalent or non-covalent (electrostatic) interactions that do not perturb the duplex structure to any great extent (Fig. 11).[112]

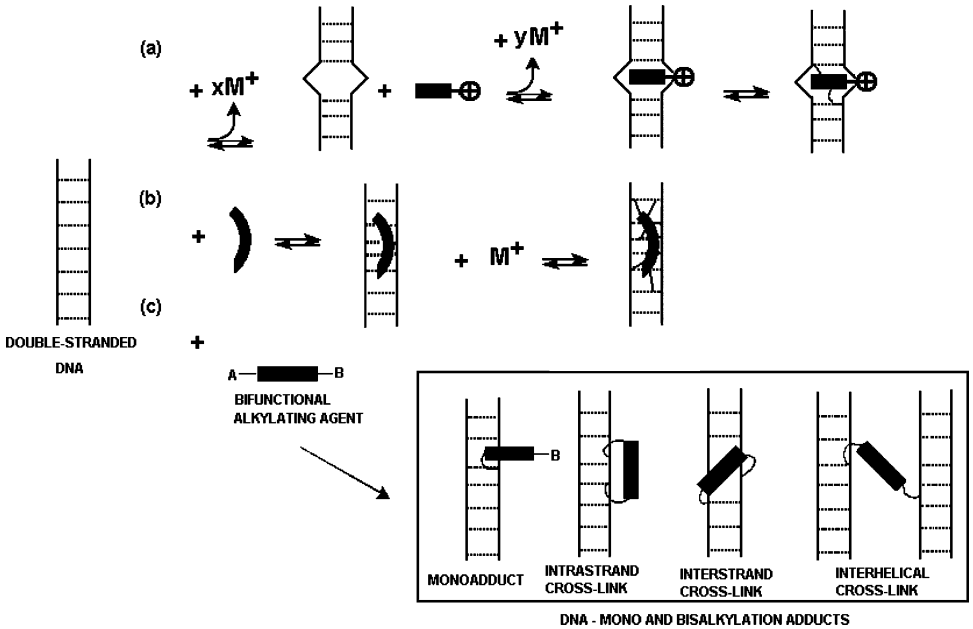

Fig. 11 Mechanistic pathways for DNA functionalisation by intrastrand, interstrand, and interhelical cross-linking, intercalating and groove-binding agents. A and B represent electrophilic moieties within the agent of interest. Adapted with permission from J. B. Chaires, A thermodynamic signature for drug-DNA binding mode, *Arch. Biochem. Biophys*., 2006, **453**, 26–31, Copyright 2006, Elsevier; and with permission from ref. 106. Copyright 1998, American Chemical Society.

Concerning DNA as a target, it is generally agreed that the most toxic of all alkylating events are those leading to inter-strand cross-links (Fig. 11).[106,111–112] Organic DNA inter-strand cross-linking agents comprise an extremely important class of clinical agents, typically exemplified by the antibiotic and antifungal bioreductive agent, mitomycin C, Fig. 12.[110] Its one-electron reduction gives rise to the semiquinone, while the hydroquinone is formed from the capture of two electrons and two protons. In hypoxic cells, DNA-interstrand cross-linking is observed, while in the presence of oxygen, redox cycling appears to be the main mechanism of action.[58,106] The electro-chemistry of mytomicin C has been reported, corroborating all the cited aspects. The radical-anion products of mytomicin C closely resembled the profile of metabolites generated from reduction with purified flavoenzymes.[113]

Bleomycins are an important class of anticancer compounds which also act *via* reductive activation and DNA binding, although in this case a quinone is not implicated. The most

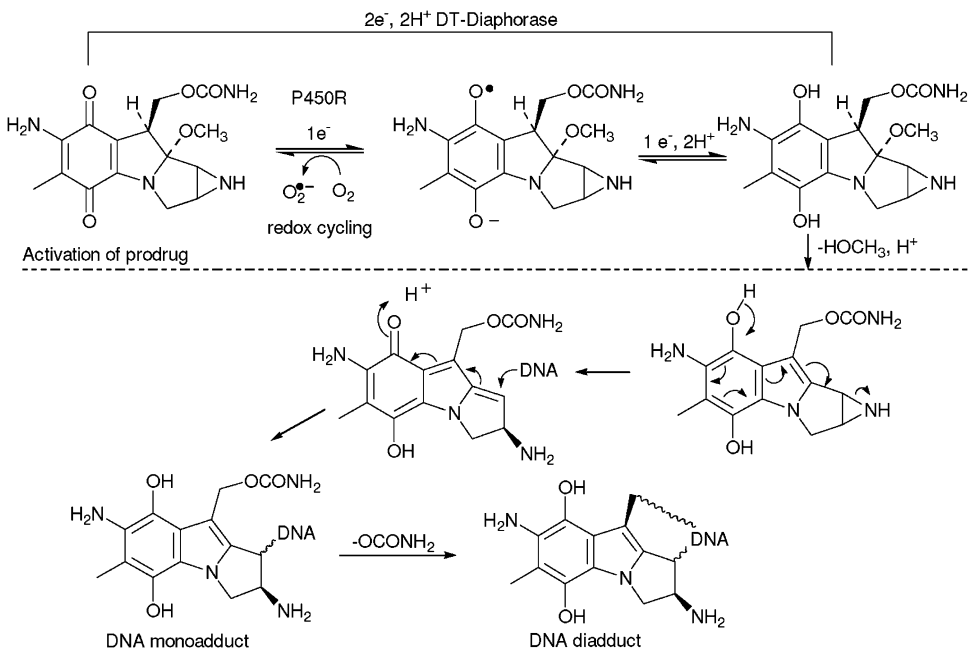

Fig. 12 Mechanism of anticancer activity of mytomicin C. Adapted with permission from ref. 110. Copyright 2002, American Chemical Society.

abundant compound in the commercial preparation, bleomycin A_2, has been found to induce DNA cleavage in the presence of Fe and O_2. Bleomycin, a large, complex natural product, possesses a metal binding zone and a DNA binding zone, and the inactive prodrug assembly has been described as O_2–Fe(II)–BLM. Although inactive, this assembly is short-lived ($\tau_{1/2}$ = 6 s at 2 °C), and is reduced to the relatively stable HOO–Fe(III)–BLM, a species referred to as ''activated bleomycin''. This compound then reacts with DNA to produce Fe(III)–BLM and DNA cleavage products.[110]

4.3.1 DNA biosensors. It is clearly of fundamental importance to explore the factors that determine the affinity and selectivity of DNA-binding compounds in order to ascertain the nature and potency of such molecules, particularly with respect to their ability to cause DNA damage. In this context, the need for stable, low cost, and readily adaptable analytical tools for the detection of DNA damage has been one of the driving forces in the development of DNA-biosensors.[114,115]

Among these, electrochemical DNA-biosensors employ double- or single stranded DNA (dsDNA and ssDNA) immobilized onto the surface of an electrochemical transducer to provide the molecular recognition element through which specific DNA-binding processes may be assessed by the electrode. The interaction of an analyte (drug, pro-drug or *in situ*-generated intermediate) with dsDNA may lead to the rupture of hydrogen bonds and consequential opening of the double helix resulting in increased accessibility to the constituent bases. The extent of DNA damage may be determined by monitoring the oxidation of the exposed bases by voltammetric methods.[115–117] The electrochemical characteristics of such ds- or ss-DNA-biosensors have been evaluated and it is clear that this approach can provide greater understanding of the mechanism of interaction between drugs and DNA and can also offer new insights in rational drug design, showing an interesting and interdisciplinary approach between analytical and medicinal chemistry.[115–117] For example, DNA-modified Hg electrodes were used to study the acidic derivative of mytomicin C.[117] From this study, the authors concluded that mytomicin C was covalently bonded to guanine residues, confirming the mechanism shown in Fig. 12.

Another interesting example is related to the study of adriamycin (**4**, Fig. 13).[118] Adriamycin is an antibiotic of the anthracycline family with a wide spectrum of chemotherapeutic applications and antineoplastic action. The adryamicin-dsDNA interaction has been studied electrochemically using a DNA-biosensor, which also enabled the study of the *in situ* generation of the reactive semiquinone radical of **4** in the presence and absence of molecular oxygen. In the presence of oxygen, a new current peak attributed to the oxidation of 8-oxo-deoxyguanosine was observed, demonstrating the ROS generation through the quinone and its effect on ds-DNA.[119]

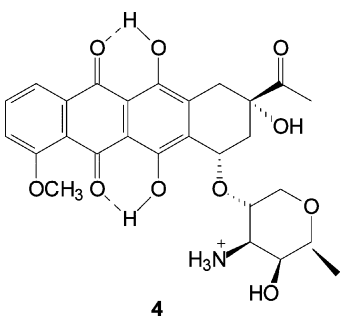

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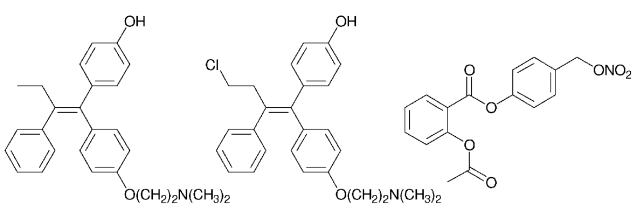
Fig. 13 Adriamycin (**4**).

In several cases, no significant interaction with DNA is apparent, suggesting an alternative target for the biological activity. In these cases, the diagnostic oxidation peaks of the bases are absent, suggesting that the integrity and maintenance of DNA conformation are conserved. This phenomenon was observed electrochemically with **3**,[120] thus paralleling the results obtained from biochemical and physicochemical experiments.[121]

DNA sensors may also show potential as tools for genotoxicity screening, which is an important factor in drug development. Genotoxicity involves reactions of molecules or their enzyme-generated metabolites with DNA, most often producing covalently bound nucleobases which may initiate carcinogenesis.[122] Films containing DNA and enzymes of nanometre thickness deposited onto electrodes can provide active sensing elements for screening the toxicity of chemicals and their metabolites including those involving oxidative stress. The basis for toxicity screening involves detection of structural damage to DNA. The most advanced genotoxicity biosensors involve arrays that incorporate many metabolic enzymes, like cytochrome *P*-450s. If the sensing devices could be designed and mass-produced to decrease their cost, they could be used at very early stages of drug development for systematic toxicity screening.[16,114,123]

4.4 Oxidative activation of phenolic pro-drugs to toxic quinonoids

Many carcinogens such as aflatoxins, polycyclic aromatic hydrocarbons and pyrrolizidine alkaloids are oxidatively activated to generate products which can alkylate DNA.[110] Some deleterious effects of non-steroidal antiestrogens, such as 4-hydroxytamoxifen and 4-hydroxytoremifene (**5** and **6**, Fig. 14) also arise from their oxidative metabolism. For example, they form QMs, although these species have been shown to be stable and to create reversible adducts with GSH, and are thus probably not directly responsible for the mild cytotoxicity of these compounds (IC_{50} MDA-MB-231 breast cancer cell line $\approx$ 28 μM).[124] Of course toxicity can also be useful in chemotherapy: the activity of such antitumour agents as cyclophosphoramide and hexamethylmelamine arises from their *in situ* oxidation and DNA alkylation. The hybrid drug NO-ASA (**7**), which consists of an aspirin molecule tethered to a nitric



5 **6** **7**

Fig. 14 4-Hydroxytamoxifen (**5**), 4-hydroxytoremifene (**6**) and *p*-NO-ASA (**7**).

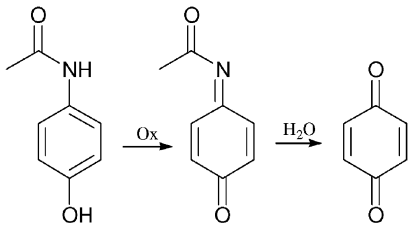


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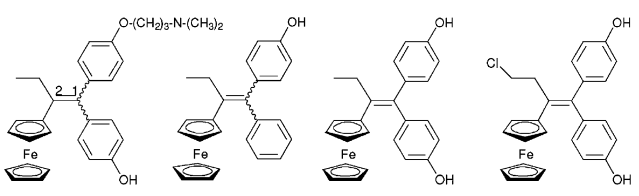
Fig. 15 Oxidative metabolism of acetaminophen (**8**) with generation of iminoquinone and further hydrolysis leading to toxic quinones.

oxide generator through a spacer, has been shown to be effective against colon cancer *in vitro* and *in vivo*. However, a recent article has shown that the cytotoxicity can not be attributed to either the aspirin or the NO, but instead to the oxidatively-generated QM which consumes GSH and disrupts the redox homeostasis.[125]

It should also be mentioned that iminoquinones can also act as alkylating agents, and this moiety has been found as a part of important metabolites after drug administration.[79] For example, the hepatoxicity associated with acetaminophen (**8**) arises from its oxidative metabolism to the corresponding iminoquinone, *N*-acetyl-*p*-benzoquinone imine (Fig. 15). However, the mechanism of cytotoxicity in the cell is still under debate, and has been attributed variously to the oxidant properties and electrophilicity of the iminoquinone.[79]

Another important class of compounds which exhibit oxidative activation is related to the bioactive catecholamines, such as adrenaline, dopamine and noradrenaline. In acidic conditions, the reversible oxidation of adrenaline to the *ortho*-quinone was observed.[126] The reversibility of the oxidation suggests that the *ortho*-quinones could then undergo electron transfer processes with the biological milieu.[127]

An example from our laboratory of the use of electrochemical experiments in the study of oxidative drug metabolism is that of the cytotoxic ferrocene (Fc)–spacer–phenol compounds, where QM formation has also been implicated. This class of compounds was originally created by the addition of a ferrocene moiety to the active metabolite of the breast cancer drug tamoxifen, in the hope of imparting a different lipophilicity and cytotoxic functionality to the existing antiestrogenic effects.[128] These compounds, called "hydroxyferrocifens" by analogy, show dual effects: (i) antiestrogenic effects on the MCF-7 breast cancer cell line, which is the standard cell line for the study of estrogen receptor (ER) interactions; (ii) cytotoxic effects on the MDA-MB-231 cell line, which does



9 *Z*+*E* **10** *Z*+*E* **11** **12**

Fig. 16 Hydroxyferrocifen, **9**, and other ferrocenyl phenols (**10–12**) which show cytotoxic effects against the MDA-MB-231 cell line. IC_{50} values (μM): **9** = 0.5 (ref. 128), **10** = 1.13 (ref. 130), **11** = 0.6 (ref. 129), **12** ≈ 1 (ref. 131).

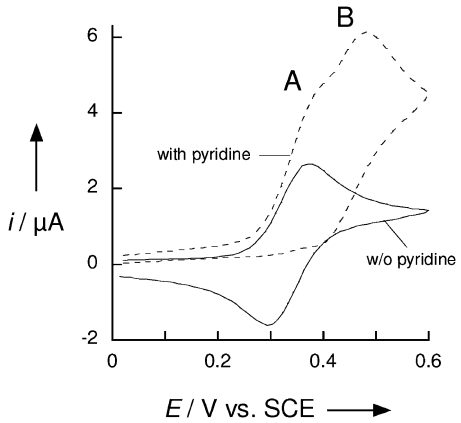


Fig. 17 Cyclic voltammograms of **11**, 2 mM in 0.1 M Bu_4NBF_4–MeOH in the absence (solid line) and presence (dashed line) of pyridine in 1 : 6 volume ratio. Scan rate 0.5 V s^{-1}. Pt electrode of 0.5 mm diameter. The phenol oxidations in MeOH occur at 0.88 and 1.17 V/SCE (not shown). Reprinted with permission from ref. 132. Wiley-VCH Copyright 2006.

not contain the ER. Fig. 16 shows the molecular structures of a hydroxyferrocifen possessing a three-carbon chain, and other similar ferrocenyl phenols (**9–12**) which also display potent cytotoxic effects.

One immediately observes that the structures of all of these cytotoxic compounds are based on a ferrocene–conjugated-spacer–phenol motif. *In vitro* cell assays over a series of similar compounds showed that each element of the motif is crucial. For example, the non-conjugated analogue of compound **11**, with an sp^3 hybridized carbon atom in place of the $CH_2{=}CH_2$ group, was significantly less cytotoxic, with an IC_{50} value of 3.5 μM.[129] Likewise, neither the non-hydroxylated analogue of **10**,[130] nor hydroxytamoxifen, lacking a ferrocene group, show any appreciable cytotoxicity.

Electrochemical studies on these types of compounds have clearly shown that the ferrocene-conjugated spacer-phenol motif gives rise to a specific oxidative mechanism, not observed in compounds lacking any element of this motif.[132] This allows us to make direct comparisons between the biological results and electrochemistry in order to obtain information about the mechanism of cytotoxicity. The cyclic voltammogram of **11** is shown in Fig. 17. In methanolic solution, one observes a reversible Fc/Fc^+ redox wave, with a higher potential, irreversible phenol oxidation wave (not shown in figure). However, when a base is added (pyridine in the reported studies), the ferrocene oxidation becomes irreversible, enhanced in intensity, and a second irreversible oxidation wave occurs at slightly higher potential, while the original phenol wave disappears.

These results suggest that, at some point after the oxidation of the ferrocene moiety when pyridine is present, there is an electron transfer from the phenol group to the ferrocenium, re-reducing it to ferrocene, which initiates the complete oxidation of the phenol moiety into a QM. This interpretation accounts for the loss of reversibility of the ferrocene oxidation (as is observed in absence of base), and the major cathodic shift of the phenol oxidation since it proceeds through intramolecular redox catalysis mediated by the Fc/Fc^+ couple. The ultimate

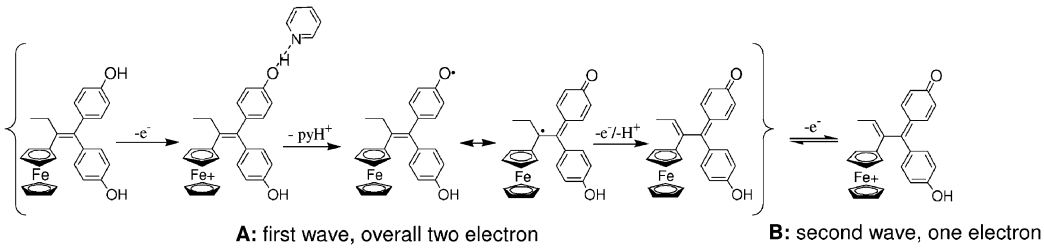


Fig. 18 Proposed mechanism for activation of compound **11**, and, by analogy, compounds **9**, **10** and **12**. In the presence of pyridine, **A** = reaction occurring at the first wave (two electrons); **B** = reaction occurring at the second wave (one electron).

product of the overall two-electron first oxidation wave, A, is then a QM structure, as shown in Fig. 18, attached to a ferrocene. The second wave, B, presented some sign of reversibility and is monoelectronic. It features the one-electron oxidation of the ferrocene moiety within the QM structure. Note that this process is slightly shifted anodically *vs*. the wave observed in the absence of pyridine, due to the electron-accepting effect of the QM moiety.

4.5 Interaction with other endobiotics

Glutathione (GSH), a sulfur containing tripeptide, is important in the regulation of the nuclear matrix organization, maintenance of cysteine residues on zinc-finger DNA binding motifs in a reduced and functional state, chromosome consolidation, DNA synthesis, DNA protection against oxidative stress, and protection of DNA-binding proteins.[6] It is also extremely important in cellular detoxification. For example, GSH removes the aforementioned toxic acetaminophen metabolites from the body *via* a Michael addition in the liver; acetaminophen toxicity has been directly linked to a depletion of these GSH reserves.[79] Adduct formation between quinones and GSH can be followed by cyclic voltammetry, as shown in Fig. 19 for the reaction between naphthoquinone and a thiol.[133] The intensity of the cathodic wave decreases after each addition, reflecting the diminishing amount of the oxidised quinone in solution.[133]

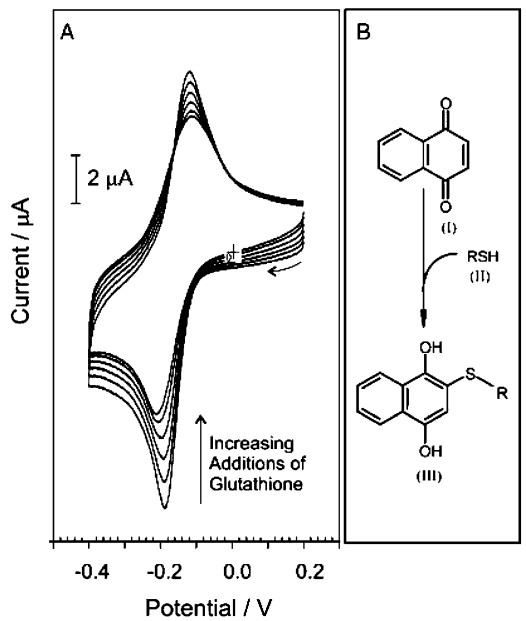


Fig. 19 (A) Response of napthoquinone (1 mM, pH 7) to increasing additions of glutathione (25 μM). (B) Reaction scheme.[133] Reproduced by permission of The Royal Society of Chemistry on behalf of the Centre National de la Recherche Scientifique.

5 Single cell experiments

Many important factors must be considered in the mechanistic aspects of *in vivo* drug activity, *e.g*., stereochemistry, diffusion, solubility, metabolism, membrane permeability, *etc*. Other parameters, like bioavailability, partition coefficients and specific enzyme interactions, also play critical roles.[24] Measurements are generally performed with model systems under conditions that are not likely to be realized intracellularly, for example, neglecting special reactant concentrations and microenvironments which can be found in the cell. Therefore the resulting conclusions are only suggestive of potential pathways of toxicity, and *ex vivo* cellular studies are highly desirable.[134]

Amperometry at platinized carbon microelectrodes has been established as an easy method for quantifying and analyzing the nature, magnitude and kinetics of the bursts of reactive oxygen and nitrogen species released by human immune cells[135,136] and skin cells.[137] The biomedical relevance of these bursts concerning the initial oxidative mechanism of skin carcinogenesis[136,138] has been demonstrated by this method. Based on an ''artificial synapse'', this method obtains its excellent sensitivity through minimization of the distance, and consequently of the solution volume, confined between the microelectrode detecting surface and that of the living cell. This allows high local concentration rises even after an extremely minute release of electroactive species by the cell (*viz*., down to the zeptomole level).[137]

In the framework of this review, we wish to focus on the electrochemical investigation of the effects of β-lapachone (**3**) treatment on oxidative bursts released by single immune cells, macrophages, using platinized carbon fibre ultramicroelectrodes. Murine macrophages were deposited and cultured in Petri dishes, incubated for different times in presence of several concentrations of **3**, and their responses were analyzed. The results show that the effect of **3** on oxidative bursts is versatile: it can enhance (''pro-oxidant'' activity) or decrease (''anti-oxidant'' activity) the release of RONS depending on the incubation time and concentration, as shown in Fig. 20(a) and (b). This observation may explain the various contrasting effects reported in literature.[95–99,139] Similar variations in the profile of RONS release are not uncommon, having been

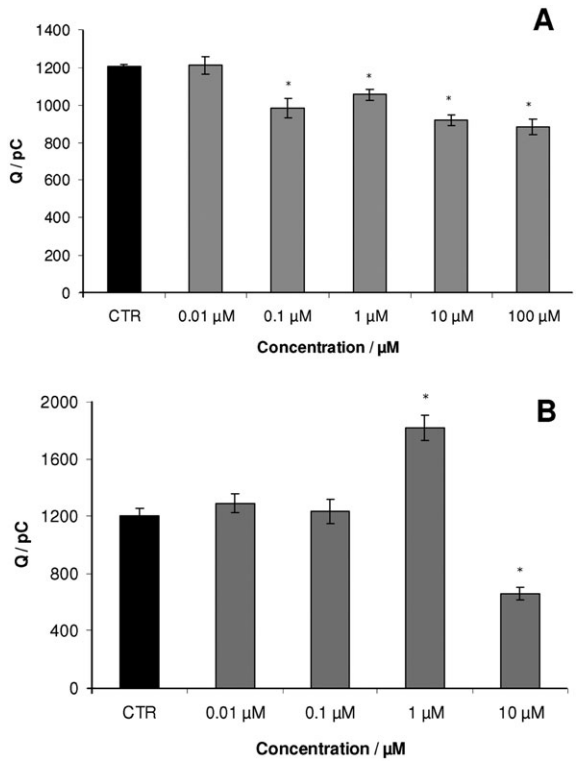


Fig. 20 Mean charge of the amperometric spikes relative to the quantity of RONS released by macrophages treated for (A) 1 h and (B) 4 h with different concentrations of **3**. Measurements were conducted on a platinized carbon fiber microelectrode at + 850 mV *vs.* SSCE, in phosphate buffer PBS (pH = 7.4). The number of cells is >30. Bars represent standard errors. $*p < 0.05$.

reported even for Vitamin C, and recently confirmed by the same electrochemical methodology as applied to macrophages depending on their activation status.[140]

Fig. 20(a) shows that after one hour of incubation, the presence of 0.1 to 100 µM of **3** led to a decrease of RONS, compared to the control (CTR). Compared to literature reports,[141] the observed effects after one hour with **3** could be explained by a possible complexation of intracellular calcium as evidenced for *ortho*-quinone derivatives. This may surely induce a decrease of the activity of calcium dependent enzymes such as constitutive NO synthases or NADPH oxidase, and then of the production of ROS and RNS, although this hypothesis is still currently under investigation.

It has however been reported that **3** induces apoptosis in cancer cell lines after incubations lasting several hours.[142] In order to enlighten this hypothesis, another series of experiments were performed with macrophages treated with longer incubation times (4 h and more) in the presence of 0.01 to 10 µM of **3**. As presented in Fig. 20(b), it is possible to observe an increase of the whole quantity of ROS and RNS released under these conditions. This increase is in agreement with reports in the literature,[143] concerning the effects on tumour cells of **3** showing that this compound exerts pro-oxidant activities after long incubation (4 and 6 h) and eventually becomes toxic even for immune cell lines at 10 µM (Fig. 20(b) and 21).

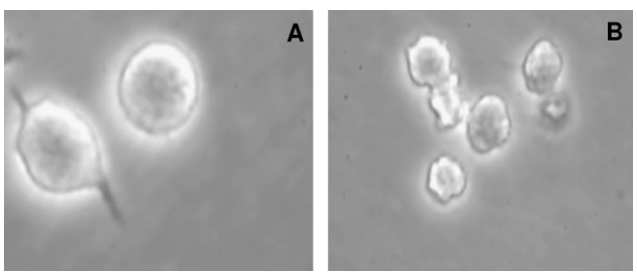


Fig. 21 Microscopic observation of control (A) and treated macrophages (RAW 264.7) with 10 µM of **3** during 4 h (B).

Reports have indicated that many of the biological activities of the quinones are centred on the *ortho*- or *para*-quinonoid moiety, which in many cases accept one and/or two electrons (redox cycling) to form *in situ* reactive oxygen species (ROS), thus favouring operative intracellular oxidative machineries,[144] which may then cause damage to several cell components.[41,55,61] The redox cycling and oxygen activation leading to increased levels of ROS is undoubtedly closely related to the quinone redox potential,[1,24,77,127,145] as substantiated for many quinones. Cell death is then expected to ensue due to the increase of the production of reactive oxygen species, especially hydrogen peroxide and peroxynitrite. The former may then engage in Fenton chemistry to produce $OH^{\bullet}$, which is very reactive towards biological cellular components. The latter is also supposed to regenerate potent hydroxyl radicals in lipophilic environments after its protonation ($pK_a = 6.8$).[139]

The amperometric analysis performed on single macrophages in this study confirmed quantitatively some of the pharmacological effects of **3** with respect to the production of ROS and RNS. These studies demonstrated the advantage of electrochemical methods for analyzing in real-time and quantitatively the effect of pharmacologically active compounds in cells on cellular oxidative bursts. Assessment and identification of RONS are usually based on reactions with various molecular probes that are oxidatively modified to generate luminescent or fluorescent signals,[146] such as 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA)[147] which is able to cross cell membranes and then, becomes trapped intracellularly as a result of deacetylation by intracellular esterases. It is becoming increasingly clear, however, that these assays lack specificity. Recent work[148–150] suggests that a series of free radical chain reactions with DCFH in the presence of peroxidase (and even in the absence of H_2O_2) may give rise to "artificial" DCF-dependent fluorescence and O_2 consumption.

6 Final considerations

Electrochemical methods (analytical and preparative) and electrochemical (thermodynamic and kinetic) parameters have shown to be extremely useful in biomedical chemistry, especially because they furnish an enormous amount of qualitative and quantitative evidence regarding the mechanisms of biological electron-transfer processes. The high versatility of electrochemical methodologies allows the mimicking of a large spectrum of biological environments, since the experimental conditions can be widely varied in the attempt to resemble them. Different ranges of pH, oxygen concentration and

solvents of diverse chemical and physicochemical properties can be used. Electroanalytical methods are accurate and precise so that their use may range from routine analyses to advanced studies involving drug activities at nanomolar concentrations. Kinetic and thermodynamic electrochemical data thus gathered may be used to help drug design as well as for screening natural biologically active compounds. As new, efficient, and low-cost drugs are urgently required in the field of neglected tropical diseases, these advantages of electrochemistry and the low-cost of the equipment compared to many advanced spectroscopies, validate and reinforce the importance of electrochemistry in this field of research. In electrochemistry, considerable progress has recently been made in the development of new and rather sophisticated techniques. The field of Biomedical Chemistry should, naturally, take advantage of this progress. The examples herein presented illustrate how electrochemical, biochemical and medical knowledge can be integrated to develop elegant strategies into the design and development of selective chemotherapeutics, yet a long way still remains. For example, it has not been possible in the present review to always provide clear answers or report firmly established electrochemical-based strategies for drug design and developments. Nevertheless, it is hoped this contribution will stimulate wider interest in this connection.

Glossary

Aflatoxins: a series of structurally related toxins produced by the fungi of genus *Aspergillus*, which grow on grains and nuts, and which can cause acute necrosis, cirrhosis, and carcinoma of the liver.

Alkylation: the transfer of an alkyl group from one molecule to another, often used to refer to the covalent binding of a molecule to DNA.

Amperometry: an electrochemical technique where a fixed electrical potential is applied to a solution containing an electroactive analyte; the current response, arising from the oxidation or reduction of the analyte, is often used to determine analyte concentration.

Antineoplastic: a general term describing drugs that inhibit the development of tumors.

ATP: the nucleotide adenosine 5′-triphosphate, which releases energy during dephosphoralation to drive many biological processes.

Catalase: a ubiquitous enzyme which catalyses the decomposition of hydrogen peroxide to water and oxygen.

Catecholamines: hormones related to the amino acid tyrosine, the most important being epinephrine, norepinephrine and dopamine.

Cyclic voltammetry: an electrochemical technique where a dynamic voltage is applied to an analyte solution and current is plotted versus the applied voltage, providing time dependent characterization of a redox-active system.

ESR spectroscopy: electronic spin resonance spectroscopy, an instrumental technique used to characterize compounds possessing unpaired electron spin(s), *i.e.* paramagnetic compounds.

Flavoenzymes (flavoproteins): a family of oxidoreductase enzymes that catalyse a wide variety of reactions and contain flavin as a cofactor.

Glucuronidation: the linkage of a molecule of glucuronic acid (essentially a glucose molecule where one of the hydroxyl groups has been oxidized to a carboxylic acid) to a biological molecule, which increases the water solubility of the latter for excretion *via* the urinary system.

Glutathione peroxidases: an enzyme family which protects the body from oxidative damage *via* the reduction of lipid peroxides and hydrogen peroxide to alcohols and water.

GSH/GSSH: the reduced and oxidized forms of the tripeptide glutathione, respectively. Glutathione is usually found in the reduced state in living cells at a concentration of around 5 mM. GSH acts as an electron donor to stabilize radical species. Upon GSH oxidation, it usually dimerizes to form GSSH, which is reduced to GSH by glutathione reductase. A large concentration of GSSH, in relation to GSH, is a marker of oxidative stress.

NAD^+/NADH: the oxidized and reduced form of nicotinamide adenine dinucleotide, a co-enzyme which shuttles electrons from one reaction system to another during cellular metabolism.

$NADP^+$/NADPH: with similar role to that of NAD^+/NADH, the oxidized and reduced form of nicotinamide adenine dinucleotide phosphate.

Nernst law and standard potential: it defines the ratio between oxidant (Ox) and reductant (Red) concentrations at the equilibrium of a redox reaction with standard potential E^0 when the solution potential is imposed at value $E = E^0 + (RT/nF)\ln([Ox]/[Red])_{eq}$, where n is the number of electron(s) exchanged in the redox reaction ($Ox + ne^- \rightleftharpoons Red$), F is the Faraday (ca. 95,600 coulombs/mole), R the gas constant and T the absolute temperature. The standard potential is that when $[Ox] = [Red]$ at equilibrium under standard thermodynamic conditions.

Overpotential: not all electrochemical reactions may be driven at equilibrium by an electrode placed in a solution because kinetics may not be fast enough compared to the rate of transport of molecules to or from the electrode active surface, or because part of the electrical potential is dissipated for ohmic heating of the solution by the electrochemical current. Ohmic overpotential makes always the reaction more difficult than predicted by the standard potential. Kinetic control by the electron transfer kinetics at the electrode surface (slow charge transfer regime) also makes the reaction more difficult because a significant part of the electrical potential is sued to overcome the electron transfer activation barrier and cannot serve for thermodynamics. Follow-up reactions (*viz.* which consume the product of electron transfer) makes the reaction more easier than predicted by E^0 since it depletes continuously the concentration of Red so that Le Chatelier principle applies. In a practical case, it is difficult to ascertain qualitatively which type of overpotentials is involved within an experiment. Since, overpotentials may span up to 500 mV or 1 V so one needs to be cautious about inferring any thermodynamic conclusion about the location of on electrochemical wave. Yet, the same problematic (except for ohmic potential) occurs also in homogeneous solutions, so the difficulty is not specific to electrochemistry.

Peroxysome: a eukaryotic organelle possessing enzymes which rid the cell of toxic substances, generally through oxidation reactions, such as the oxidation of fatty acids.

Reference electrode: since (i) only voltage differences may be measured, and (ii) only the electrical potential difference between an electrode and the solution in which it is immersed may be used to drive an electrochemical reaction, electrochemists report the active electrode potential versus that of an inert electrode which is at any moment in equilibrium with the solution and does not deliver any significant current flow. Such an inert electrode is termed a reference electrode. Many reference electrodes exist and may be used according to the nature of the solution or other requirements. The ideal reference electrode as defined by IUPAC is the normal hydrogen reference electrode (NHE), based on the $H^+/(1/2H_2)$ couple with $E^0 = 0$ V by definition. Yet since this electrode is hardly usable in real experimental situations, electrochemists use other reference electrodes whose potentials are constant *vs.* the NHE. Among those the most popular are the SCE (saturated calomel electrode: $Hg_2^{2+} + 2e^- \rightarrow 2Hg$ where Hg_2^{2+} concentration is fixed by saturation of calomel) and the SRE one (silver reference electrode, based on $Ag^+ + e^- \rightarrow Ag$ where Ag^+ concentration is fixed by saturation of AgCl).

Reversible and irreversible electron transfer: a reversible electron transfer is a pure electron transfer without any preceding or following chemical reaction associated to it within the time scale of the experiment. When the electron transfer is fast, it then obeys perfectly the Nernst law in absence of ohmic distortions (see above: ''Nernst law'' and ''overpotential'') and is called ''reversible''. When the electron transfer is not fast so that its kinetics affect the measurement the electrochemical wave is termed ''slow and chemically reversible''. Conversely when the product of electron transfer evolves chemically faster than the experimental time scale, the electrochemical wave is called ''irreversible''.

Triatomine: bloodsucking insects found in Latin America and the southern U. S. which are important in the transmission of *Trypanosoma cruzi*, the parasite that causes Chagas disease in humans.

Trypanothione: a thiol peptide, similar to glutathione, found in parasites such as leishmania and trypanosomes, the latter which are responsible for leishmaniasis, sleeping sickness and Chagas' disease.

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