Network theory and the resilience of redox signaling

John T. Hancock[1*], David Veal[1], Annabel Laura Hancock[2]

1. Department of Applied Sciences, University of the West of England, Bristol, UK.
2. Centre for Medieval Studies, King's Manor, University of York, York, UK.

[*]Correspondence:
Prof. John T. Hancock
Faculty of Health and Applied Sciences,
University of the West of England, Bristol, BS16 1QY, UK.
john.hancock@uwe.ac.uk

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ABSTRACT

The redox status inside and around cells is critically important to control, being used to maintain reduced compounds in the correct state and for cell signaling mechanisms. A myriad of compounds and proteins are involved in a vast network system to regulate the redox state of biological systems. These include reactive molecules such reactive oxygen species (ROS), nitric oxide ($NO^{\cdot}$) and hydrogen sulfide (H_2S) along with systems for their removal, such as antioxidants. Redox buffering involves molecules such as glutathione, low molecular weight thiols and ascorbate. Network Theory attempts to give the mechanisms underlying complex

networks a mathematical and model-based underpinning and it has been suggested that metabolic systems can be described as scale-free networks, having a power law degree distribution. Such networks are said to be both robust but vulnerable, suggesting a level of resilience. Redox metabolism also has to be robust, being maintained in what has been described as the Goldilocks Zone, while it is also vulnerable to outside influence, often leading to the phenomenon referred to as oxidative stress. Therefore, it is suggested here that a holistic approach to understand redox networks should embrace Network Theory, which may be able to predict characteristics of the redox network that can be targeted for new therapeutics or agricultural treatments.

KEYWORDS | Hydrogen sulfide; Nernst equation; Network Theory, Nitric oxide; Reactive oxygen species; Redox; Resilience, Signaling; Stress.

ABBREVIATIONS | APX, Ascorbate peroxidase; Arg, arginine; CAT, catalase; cGMP, Cyclic guanosine monophosphate; Cys, cysteine; Cys-Gly, cysteinyl-glycine; cySS, cystine; DHA, dehydroascorbate; EDRF, endothelial-derived relaxing factor; FA, fatty acid; FA-NO, nitro-fatty acid; Glu, glutamate; γ-Glu-Cys, γ-glutamyl-cysteine; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; LMW, low-molecular weight; MPO, myeloperoxidase; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; α-TCP, α-tocopherol; TRX,

thioredoxin; TRX-red, thioredoxin reductase; X/HX, xanthine/hypoxanthine; XO, Xanthine oxidase.

CONTENTS

1. INTRODUCTION

The redox status of the cell is extremely important and well controlled [1]. It is vital to be maintained in a relatively reduced state so the cell may maintain the reduced versions of cofactors such as NAD(P)H [2], is important to allow chemical interactions, and is allowed to fluctuate to facilitate its role in cell signaling [3,4]. The redox of a cell will be influenced by a range of factors including the arrival of redox active compounds from the outside [5], the production of intracellular redox compounds such as glutathione [6] or the activity of a range of antioxidant mechanisms [7].

Life evolved in an environment very different to that which most organisms are exposed to today [8]. Early life was influenced by sulfur chemistry [9] and later organisms had to manage the increase in atmospheric and dissolved oxygen [10]. Many of the compounds influencing evolution exist as redox couples and therefore have the ability to impact on the redox state of the solution in which they exist. Cells had to develop strategies to manage the presence of such redox couples, some of which would be oxidizing to the cellular environment. Interestingly, cells not only tolerated the presence of such redox couples but also adopted many of these redox compounds to be regulators of cellular activity [8,11-13]. This included compounds such as hydrogen peroxide (H_2O_2), hydrogen sulfide (H_2S) and nitric oxide ($NO^{\cdot}$). Therefore, one constant during evolution, and in present day, is that redox chemistry is vital to the survival of cells, tissues and organisms.

It is important, therefore, that the way redox chemistry influences cell activity is more fully understood, especially on the arrival of redox active compounds from the environment or other cells [14]. Here, it is suggested that network theory can be used as an aid in the understanding of how the redox status of the cell may alter

when impacted upon by external factors or by cellular dysfunction. Hence, as a results it will help the understanding of cellular adaptation and resilience, which will be key to healthier crops and disease management in animals, including humans.

2. REDOX BIOLOGY AND THE CONTROL OF CELLULAR FUNCTION

Early work on redox active signaling molecules focused mainly on those centered around oxygen-based chemistry. Molecules such as the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide were found to be produced by cells and important for pathogen resistance [15] but more recently it has been found that such molecules have a positive role in controlling cell function. Enzymes such as the NADPH oxidase family [recently reviewed in 16] and peroxidases [17] are involved.

However, ROS are not the only redox active compounds that can influence cellular function. In 1987 it was realized that nitric oxide (NO) has a major role [18], being previously dubbed as endothelial-derived relaxing factor (EDRF), and later found to be produced by nitric oxide synthases (NOS) [19]. This opened the door to much research into how reactive compounds can be involved in cell signaling processes. This included NO [20] and ROS [21], but also hydrogen sulfide (H_2S) [22] and hydrogen gas (H_2) [23,24]. However, the generation of this suite of compounds can be initiated by common triggers and they may temporally and spatially accumulate together, suggesting numerous interactions between them [25,26]. The notion of a Reactive Species Interactome has been mooted, suggesting that the redox components in a cell form a regulatory system that can overcome environmental challenge and stresses, and may lead to novel approaches to personalized medicine [27].

Compounds such as ROS exist as redox couples and therefore can have an influence on the redox poise of the cell. The intracellular redox status is kept at a very reducing level. This is partly to enable critical compounds such as NAD(P)H to be maintained in a reduced state [2], but also to enable redox signaling [3]. Several groups have attempted to measure the extracellular [28-30] and intracellular redox states [31-35] while it is well recognized that the redox of the cell is heavily buffered, partly by glutathione [36]. Using the glutathione couple (ie GSH/GSSG) as a model with the aid of the Nernst equation it is possible to calculate the cell's redox state once the concentrations of GSH and GSSG have been determined [36-38] (the mid-point potential for the GSH/GSSG couple has be reported [39]). However, as it requires two GSH to create one GSSG the Nernst equation becomes a squared relationship – the corollary of this is that the intracellular redox also depends on the total GSH+GSSG content, which can change [40]. In plasma, a diurnal pattern has been seen [28], while it has also be suggested that redox status may follow the circadian clock [1]. However, using such measurements the intracellular redox has been estimated to be around -242mV (relative to hydrogen), although more negative numbers have also be reported [37,41]. Significantly, it has also be suggested that it can become significantly more oxidizing leading to the onset of cell differentiation or apoptosis [36], while plasma redox has been found to become more oxidizing with age [1,33], and is therefore less adaptable. However, several other redox buffers also exist in cells, including other low molecular weight (LMW) thiols, such as cysteine (Cys), cyteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteine (γ-Glu-Cys) [42]. Another major redox buffer is ascorbate, while cells rely on a range of other antioxidant compounds (some soured from diet) and antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT).

Therefore there is a network of reactions between a range of reactive compounds in cells which may impinge on the overall redox state of the cell. On top of this many such compounds may arrive at a cell from the environment and it is thought that the arrival of oxidizing compounds can lead to oxidative stress [43] and the harmful downstream effects that causes disease [44].

The background redox status and numerous redox couples feeds into the signaling network of the cell. NO, for example, can activate soluble guanylyl cyclase and so increase intracellular cGMP levels [45], leading to downstream effects. Of particular importance is the post-translational alteration of protein thiols. ROS, NO, H_2S and glutathione can all lead to modifications of thiols, perhaps in a competitive manner [25,46]. However, the capacity to undertake such reactions will also be influenced by the redox environment of the thiol group and therefore the cellular redox status is important. Proteins which can be influenced by thiol modification includes those involved in metabolism, such as glyceraldehyde 3-phosphate dehydrogenase [47], transcription factors [48], and phosphatases [49], the latter impinging on overall phosphorylation levels in cells. For a more comprehensive review on redox signaling see [50,51].

What is clear from the literature is that controlling the redox status in a cell within limits is important [36] and the redox status of the cell needs to be resilient to influences from outside, such as the arrival of redox active compounds (eg ROS, NO, H_2S etc). Here, it is mooted that network theory may in the future be able to model how redox poise might change in cells.

3. THE BASICS OF NETWORK THEORY

Network Theory is an attempt to place a mathematical underpinning to complex systems. One of the most influential papers in this field was published in 1999, when Barabasi and Albert introduced the concept of scaling in random networks [52]. The idea of the use of Network Theory was popularized by the publication of *Linked* [53], with a more recent book, *Network Science*, published by the same author [54].

Many of the networks which have been used to describe a variety of systems, from social, technological to biological are what are described as scale-free networks. Li *et al*. [55] recently has suggested that the term is not defined rigorously enough but there are some features which are useful for redox metabolism. Firstly, such networks are described as having a series of nodes which are connected. This could be visualized as shown in Figure 1. Secondly, such networks follow a power law degree of distribution (Figure 2). That is, it would be predicted that most of the nodes have only a few links to other nodes, but there are fewer which are highly connected and can be thought of as hubs. Such hubs would have a major influence on the whole. In biological systems the nodes and hubs are determined by the presence of certain chemicals, while the interconnections (edges) are the processes which convert one chemical to another (enzyme activities, for example) [56]. In this way, metabolic processes can be re-drawn in the ball and stick manner seen in Figure 1, and as discussed for redox biology below.

Of particular relevance to the discussion below on redox resilience, scale-free networks are said to be “robust yet fragile” and have “error tolerance but attack vulnerability” [55,57]. This means that there is a built in resilience to change, especially if a node is altered, but there is less resilience if a major hub is modulated.

Therefore, if this is going to be used to help in our understanding of redox mechanisms the nodes and hubs need to be determined (see below).

Scale-free networks have not been short of criticism. It has been argued that elements of biochemistry have been misinterpreted when scale-free Network Theory has been applied [58] and therefore scale-free networks do not provide a good model. In their paper, Li *et al*. [55] argued that in biology the influence of evolution, design, functionality and other constraints should not be ignored, but are not readily taken into account in a scale-free network. One of the constraints is that biological systems have a structure: cells are not uniform but are compartmentalized, often in ways which are not obvious. Even the cytoplasm is not uniform and compartmentalization has been reported and discussed for many signaling mechanisms [59], including those involving cAMP [60,61], Ca^{2+} [62,63], ROS [64,65] and redox poise [14,66]. Therefore, any model needs to take such structures into account, but scale-free network models may not do this. Although not concerned directly with the physical structures of cells, Itzkovitz *et al*. [67] suggested how to incorporate local structures of networks in what was referred to as course-graining, which was an attempt to understand the microscopic features of networks and their structures, referred to as motifs. They looked at signal transduction in cells, in particular the MAP-kinase pathways. Further work by others was carried out, taking the idea of structural patterns to understand complex networks such as found in biological systems [68]. Their models were based on complexity signatures, a suggested biological example is species density in a rain forest.

Scale-free networks are not the only mathematical underpinning which need to be considered, and in fact their use has been criticized in recent papers [55, 69,70]. Another approach, discussed by Li *et al*. [55], is referred to by the acronym

212 HOT, which stands for Highly Optimized Tolerance [71,72] or Heuristically Organized
213 Tradeoffs [73]. Introduced from systems in physics, it concentrates on defining
214 organised complexity. As it has a focus on constraints, including function and
215 organisation it may be considered better for the modelling of biological systems, and
216 in fact has been used to model cell-cell architectures [74]. Interestingly, HOT
217 networks have been considered to give opposite results from scale-free networks
218 [55]. Furthermore, HOT is a predictive model which can model how external factors
219 may change the network. Therefore, it may be useful for modelling oxidative stress
220 which can be initiated by the arrival of oxidant molecules from outside of the cell.
221 Other models for modelling metabolism have also been used, for example by
222 Pearcy *et al*. [75] looking at bacterial metabolism and Toubiana *et al*. studying plant
223 metabolism [76].
224
225 **4. CAN NETWORK THEORY BE USEFUL TO EXPLAIN REDOX BIOLOGY?**
226 The idea of using Network Theory to get a better understanding of biological systems
227 is certainly not a new idea. In 1925, Yule [77] developed models to study species
228 within genera of plants. To study mutants in bacterial populations models were
229 developed by Luria and Delbrück in 1943 [78]. Some of the early work is further
230 discussed by Mandelbrot [79] and Li *et al*. [55]. More recently several papers have
231 used Network Theory to explain metabolic, genetic and biochemical processes
232 [56,80-82] while mathematical models have been used to determine the energy cost
233 of cells sensing their environment [83].
234 Barabasi [39] suggests that metabolism can be drawn as a network, where
235 the nodes are the chemicals involved and the links are the biochemical reactions
236 which connect them. He goes on to suggest that there is a ‘cellular network’ where

all cellular components are connected by links, either reactions or physically. Having examined the metabolic maps of forty-three organisms the cellular network appeared to have a scale-free topology. Hubs of the metabolism could be seen where a few molecules were involved in the majority of reactions while most molecules only participated in one or two interactions [53]. It was suggested that despite differences in constituents and pathways, metabolic networks had many similarities to non-biological systems [84]. It was further suggested that the organization of metabolism had a design which conformed to that of an error tolerant and robust scale-free network. Furthermore, Barabasi [53] suggests that if molecules are separated by many nodes the perturbation of one will decay before effects on the other are seen, suggesting resilience of the system. But he goes on to say that cellular molecules only have three degrees of separation in general. Such a network is described as scale-free with small world properties [85]. Interestingly, they also predicted that the molecules forming the largest hubs were found earlier in evolutionary history.

However, very recently there has been doubt cast on the validity of using a Network Theory approach. Firstly the actual definition of a scale-free network [55] has been questioned (there are at least six characteristics which may be used as a definition). Secondly, their use for a variety of systems has been robustly explored [69]. This work looked at a variety of possible networks including social and technological as well as those involving, transportation and information, but of relevance here biological networks were also considered. While social networks were found to be largely weakly scale-free, some of the most strongly scale-free were found to be biological, particularly metabolic systems [69,70]. Therefore, here, we suggest, even though there are other models [71,73,75,76] that using Network

Theory based on a scale-free model to look at the metabolic processes which control redox homeostasis would be a valid approach.

To use a scale-free network with a node and hub model the components of the network needs to be known, along with the mechanisms which allow their interconversion, such as chemical or enzymatic processes [39]. With respective to redox it has been suggested that there is a *Redox Code* [1]. Four principles were given: 1, a core feature is the metabolism of NAD(P)/NAD(P)H operating at near equilibrium; 2, activities can be controlled through protein-based sulfur switching; 3, activation of ROS production in a cyclic manner supports differentiation and the life cycle; 4, redox networks form an adaptive system to allow responses to the environment. These principles highlight two things. Firstly, the redox environment has to be maintained at a certain level to allow NAD(P)H metabolism to work efficiently and to allow protein thiol modification – it is known to be relatively reducing [36] which would facilitate the metabolism needed [2]. Secondly, the redox status of the cell has to be able to cope with change, either from endogenous metabolism or from external factors.

With a focus on striated muscle, it has been suggested that the redox environment is maintained in what has been dubbed the *Goldilocks Zone* [86], and as depicted in Figure 3A. It was suggested that the redox environment fluctuates, in this case because of exercise and aerobic metabolism. However, despite the variation of the redox if never falls outside a defined region, neither becoming too oxidizing nor too reducing, and hence cell function and integrity is maintained. For other cells this could also be a good model, as the redox may fluctuate (Figure 3A), for example with the movement of glutathione [40], or as a diurnal pattern [28] or with age [1] (see Figure 3B), but the redox environment will not be modulated enough to

cause oxidative or reductive stress, as schematically shown in Figure 3. This shows some resilience of the redox status, as it can be altered but will stay within safe limits, although it has been suggested that with age redox may become more oxidizing with a loss of adaptability [1]. However, if the network of redox components is altered too much redox stress will result. Network theory would be a useful tool to determine which nodes are vulnerable to attack and therefore may influence the overall redox environment.

To use the ideas of a scale-free network as proposed by Barabasi and Albert [52] and recently suggested for metabolic pathways by Briodo and Clauset [69], a node and stick model needs to be drawn. Taking elements of the possible redox components in a cell, a network such as Figure 4 can be created. However, there are several caveats here. Firstly, this would not be the same for all organisms or indeed tissues or cells. For example, there is considerable controversy over the enzymes which generate NO in higher plants which are probably not the same as those seen in animals [87], so drawing a network in this area of redox biology would not be easy. This also means that the network drawn for one system would need to be tailored for another system. Secondly, many of the components of the redox network exist in interchangeable couples, with the interchange involving the addition or removal of electrons and perhaps protons, for example, the 2GSH/GSSG couple. If the redox mid-point potential for a couple is relatively negative and near the redox status of the biological fluid it is in, then fluctuations of the redox of that fluid, as discussed by Alleman [86], has the potential to alter the concentration ratio of the components of that couple, and this can potentially alter the biological activity of that redox component. An assessment of the impact of the redox environment on some relevant redox couples has been previously reviewed [14]. Thirdly, there is a

311 considerable amount of structure in biological systems that needs to be taken into
312 account. For example, ROS can be generated by soluble enzymes (eg xanthine
313 oxidoreductase), membrane-bound enzymes (NAPDH oxidases) or from organelles
314 (eg mitochondria). It would not be unusual for redox components to move between
315 cellular compartments, for example glutathione out of the cell [40] or ROS from
316 organelles, and therefore have profound effects [88].

317 Despite the chemical and biological caveats above, all which may be
318 overcome with time, Network Theory has the potential to assess the resilience of
319 networks such as those redox components. The redox status of the cell is not static
320 [40,86] and will be influenced by external factors, possibly leading to oxidative stress
321 [43]. Many interacting redox compounds may arrive from the outside, including ROS,
322 NO, H_2S and H_2 [25,26] – the latter which has been shown to interfere with ROS and
323 antioxidant metabolism [24,89]. Therefore, to keep the redox status within the
324 Goldilocks Zone [86] an element of resilience is needed. Network Theory can assess
325 such resilience.

326 It has been found that scale-free networks have a high, unexpected, degree of
327 robustness [57]. Even with high node failure rates they are able to function, but this
328 means that they are vulnerable to attack if certain nodes are upset. Therefore, if this
329 is extrapolated to redox networks it suggests that some nodes are not as important
330 as others, that there is a redundancy in some and not others. Redundancy of
331 metabolic networks has recently been the subject of review [90]. This extrapolation
332 also suggests that there are important nodes, referred to in scale-free networks as
333 hubs, which are important and vulnerable. Therefore, within redox networks such
334 robust nodes and vulnerable hubs need to be identified. Owing to its high

concentration in cells and therefore high buffering capacity the glutathione couple [36] may be regarded as a key redox hub.

Network theory can in principle take the field further. By developing analytical tools and mathematical frameworks to scale-free networks, using examples from biological sciences, such as ecological and gene regulatory networks, Gao *et al*. [91] suggest that it is possible to define the characteristics of a network which can be used to either enhance or reduce resilience. Such characteristics could then be used to enhance redox resilience through pharmacological intervention or agricultural treatments. Perhaps glutathione metabolism is one such characteristic [92].

5. CONCLUSIONS AND PERSPECTIVES

The redox status of biological fluids is critically important, used in cell signaling mechanisms [8,11,12,13] and known to be vital for the health of the cell [93-95]. However, although the maintenance of a reducing redox state in cells seems to be a universal feature it must be remembered that the constituent players in different organisms may be different. For example, NO is almost certainly not produced by a nitric oxide synthase in higher plants, the NO being derived through a nitrate reductase-mediated route [87].

Although there are several network models and mathematical treatise [55], it has recently been suggested that metabolic systems resemble a scale-free network [69,70] and as such can be schematically represented as an interconnected node system as depicted in Figures 1 and 4. Therefore, it is mooted here that such a treatise of the redox network can be used to determine what would constitute the hubs of the system and therefore which parts are vulnerable to external influence. Within such a network it has been suggested that some nodes can be modulated

with little effect to the whole system [53] and therefore these would not make good drug targets if redox status is to be maintained. It would be the hubs of the system that would need to be the focus of future work and effects of such hubs could be determined using a scale-free network model. However, to do this, the spatial and temporal concentrations of the constituents of such nodes and hubs would need to be determined. Future therapies, making sure that redox does not have any delirious effects, would need to make sure that the redox status of cell and biological fluids remained in the Goldilocks Zone [86], and network theory may be a way to test any future potential redox-based pharmaceuticals [27] or agricultural useful treatments.

In conclusion, there is little doubt that a vast network of redox components (couples) and interacting partners coordinate to maintain the over redox environment of biological systems, and even if there are variations of redox the status is kept within a Goldilocks Zone [86]. Network Theory [69] could be used to determine the resilience and vulnerability of redox metabolism but temporal and spatial concentrations (of both sides of a redox couple) would need to be known and such detail needs to be the future focus of research which can then benefit from the mathematics being developed in the field of Network Theory.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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649

650 **FIGURES**

651 Figure 1: A theoretical network. Here the size of the nodes and the width of the lines

652 are scaled to show importance for their effect on the network as a whole.

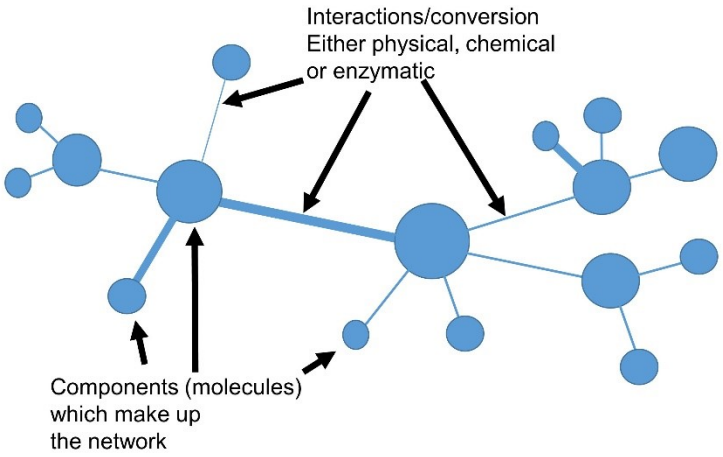

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Figure 2: The power law degree distribution of a scale-free network. This predicts that most of the nodes have only a few links, where some are highly connected and can be thought of as hubs, having a major influence on the whole.

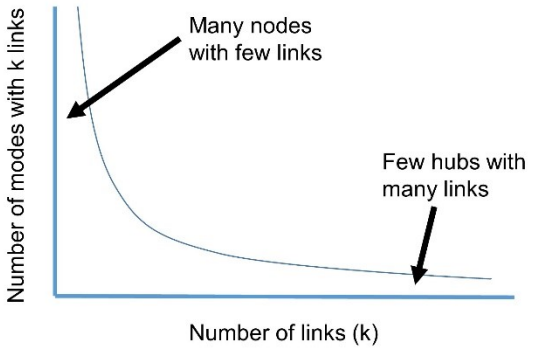

Figure 3: The redox environment of cells. A: the redox environment may fluctuate but is held in what has been dubbed the Goldilocks Zone. If the redox state moves outside this variable then cellular stress may result. Figure adapted from [86]. B: The average redox environment tends to drift towards being more oxidized with time [1,33].

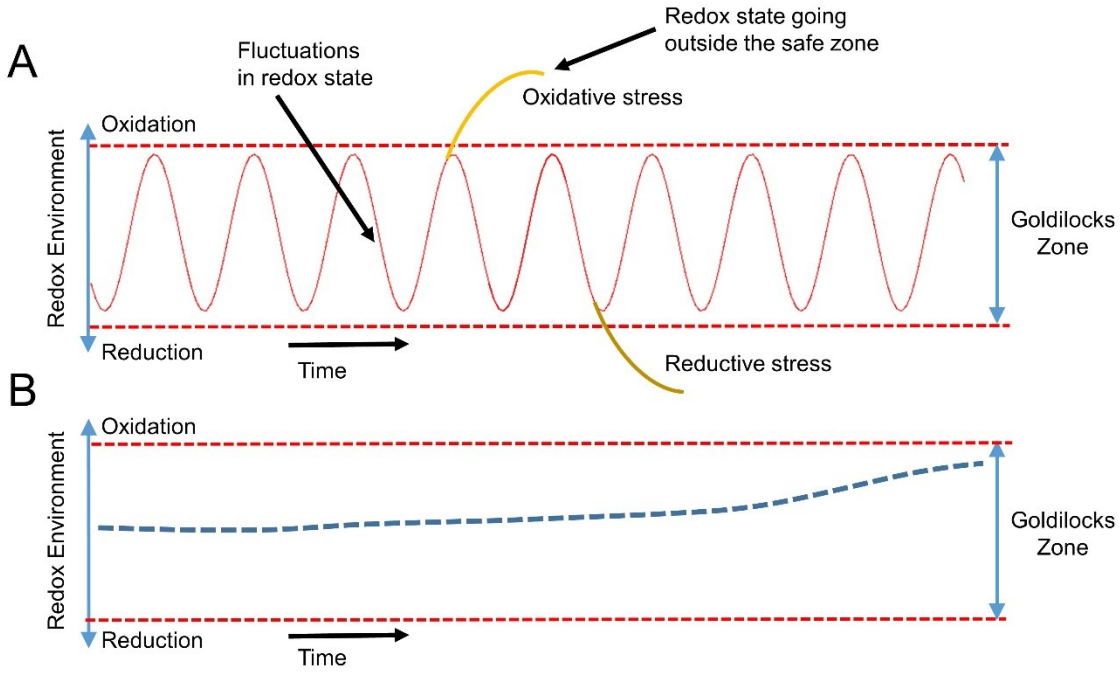

Figure 4: A node and edge approach to representing a redox scale-free network. The size of the node represents the sphere of influence that molecule may have on the overall network (not to scale). * denotes redox couple. Names in red can be considered end points for signaling. NAD or NADP in yellow indicates the involvement of $NAD(P)^+$/NAD(P)H couples. Orange solid lines denotes cellular membranes. Although the cytoplasm, mitochondria (details not shown) and the exterior of the cell are represented, the endoplasmic reticulum and nucleus is not shown even though they would have influential redox environments. Also missing are plant-specific redox components such as chloroplast and nitrate reductase. Cytoplasmic redox will be under the influence from external factors, such as ROS, RNS, H_2S and antioxidants. A holistic understanding would need a comprehensive and cell specific network, with scaling to represent measured concentrations and potential influence.