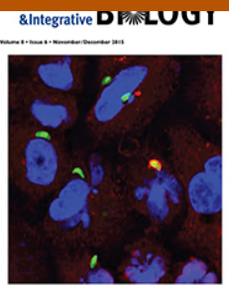

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Production and removal of superoxide anion radical by artificial metalloenzymes and redox-active metals

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Generation of reactive oxygen species is useful for various medical, engineering and agricultural purposes. These include clinical modulation of immunological mechanism, enhanced degradation of organic compounds released to the environments, removal of microorganisms for the hygienic purpose, and agricultural pest control; both directly acting against pathogenic microorganisms and indirectly *via* stimulation of plant defense mechanism represented by systemic acquired resistance and hypersensitive response. By aiming to develop a novel classes of artificial redox-active biocatalysts involved in production and/or removal of superoxide anion radicals, recent attempts for understanding and modification of natural catalytic proteins and functional DNA sequences of mammalian and plant origins are covered in this review article.

Introduction

Empirically, people have been aware that increased intake of antioxidants in the form of fruits and vegetables may reduce the risk of chronic diseases, chiefly of cancer.[1] Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular responses to xenobiotics, cytokines, and bacterial invasion; and oxidative stress in living cells of both animal and plant origins.[2] Today, natural plant products are viewed as potential candidates for the cancer chemoprevention due to their antioxidant, anti-inflammatory and antitumor activities and, therefore, intake of such antioxidants are necessary for controlling degenerative reactions produced by ROS and nitrogen species *in vivo*.[1]

On the other hand, generation of ROS is useful for various medical, engineering and agricultural purposes namely, clinical modulation of immunological mechanism,[3,4] enhanced degradation of organic compounds released to the environments,[5] removal of microorganisms for the hygienic purpose,[6] and agricultural pest control both directly acting against pathogenic microorganisms and indirectly *via* stimulation of plant defense mechanism represented by systemic acquired resistance and hypersensitive response.[7,8]

In this review article, recent approaches for understanding and modification of natural catalytic proteins and functional DNA sequences of mammalian and plant origins are covered, aiming to develop a novel classes of artificial redox-active biocatalysts involved in production and/or removal superoxide anion radicals ($O_2^{\bullet -}$).

In general, catalysts (*C*s) can be defined as the set of different types of elements, molecules or compounds as follows:

$$\{Cs\} = \{\{OCs\}, \{ICs\}, \{BCs\}\} \quad (1)$$

where *OC*s, *IC*s, and *BC*s are organic catalysts, inorganic catalysts, and biocatalysts, respectively.[9] *OC*s can be represented by biological and bio-inspired organic molecules such as guanidine-type[10] or amino acid proline-type catalysts,[11] and *IC*s can be represented by inorganic molecules or complex such as metal-based catalysts.[12] While the natures of *OC*s and *IC*s can be clearly defined and described based on their chemical properties, the category of *BC*s simply implies the origins but not the natures of these catalysts. It is trivial that the set {*BC*s} can be divided into 2 subsets as follows:

$$\{BCs\} = \{\{Es\}, \{Ns\}\} \quad (2)$$

where *E*s and *N*s are enzymes and nucleozymes, respectively.

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In fact, *Es* and *Ns* can be further confirmed as follows:

$$\{Es\} \in \{\{Cs\} \cap \{\text{proteins} \cup \text{peptides}\}\} \quad (3)$$

$$\{Ns\} \in \{\{Cs\} \cap \{\text{RNA} \cup \text{DNA}\}\} \quad (4)$$

Moreover, the sets of {*Es*} and {*Ns*} can be further divided into subsets as follows:

$$\{Es\} = \{\{E_n s\}, \{E_a s\}\} \quad (5)$$

$$\{Ns\} = \{\{N_n s\}, \{N_a s\}\} \quad (6)$$

where E_ns and N_ns, are natural enzymes and nucleozymes, respectively, which can be found in or produced by living organisms; and E_as and N_as are artificial enzymes and nucleozyme, respectively, which are now newly designed or engineered in the laboratory. Among the *Es*, many portion of both E_ns and E_as reportedly bind catalytically active metals directly or indirectly (by possessing prosthetic groups such as iron-centered hemes), to form the center of catalytic reactions within the molecules.[13] Thus, catalytic activities of natural and artificial metal-binding enzymes can be attributed to the behaviors of bound metals. Therefore, it is natural to obtain the following proposition, *P*(*Es*).

$$P(Es) = \exists \{Es\} \in \{ICs\} \quad (7)$$

This type of enzymes should be categorized and termed as metalloenzymes. According to recent reviews on metalloenzymes,[13,14] an artificial metalloenzyme can be designed *de novo* by arranging the peptidic sequence composed of 20 natural amino acids. Basically, such *de novo* designs of metalloproteins can be achieved freely designing the amino acid sequences capable of binding metal ions.[13] In order to artificially design or modify the catalytic peptides or proteins, it is much easier to learn from the catalytically active peptidic motifs within the naturally existing active enzymes or proteins as the platforms of engineering.[9,15]

As reported by Yeung et al.,[16] modification of myoglobin (Mb) is one of successful cases in engineering of semi-natural metalloenzyme. Accordingly, natural Mb was re-designed into a functional nitric oxide reductase, by newly forming a non-heme iron binding site in the distal pocket of Mb. Presence of such natural, semi-natural and artificial metalloenzymes consists the elements of conceptual subset of bio-originated catalysts within the set of {*ICs*} fulfilling the proposition (7):

$$P(Es) = \exists \{Es\} \in \{ICs\}.$$

Similarly to the cases of metalloenzymes, we have been seeking for the cases of metallonucleozymes, in which catalytic activities of natural and artificial metal-binding nucleic acids (DNAs and RNAs) can be attributed to the behaviors of bound metals. Therefore, it is also natural to obtain the following proposition, *P*(*Ns*).

$$P(Ns) = \exists \{Ns\} \in \{ICs\} \quad (8)$$

In the processes to revise recent progresses, we would like to highlight some examples of metallonucleozymes which may be supporting the proposition (8).

Two propositions listed above (7, 8) can be combined and generalized as follows:

$$P(BCs) = \exists \{BCs\} \in \{ICs\} \quad (9)$$

By analogy to the cases of metal-binding *BC*s, we can assume that catalytic actions of some *BC*s can be attributed to the catalytic mode of actions similar to *OC*s, since proline, one of natural amino acids composing proteins, is now consider as an active catalyst.[11] Thus, following proposition can be arisen.

$$P(BCs) = \exists \{BCs\} \in \{OCs\} \quad (10)$$

This proposition will be discussed at the end of this article.

Non-biological catalysts producing $O_2^{\bullet -}$

As an example of {*ICs*} in definitive proposition (1), we have studied the mechanism of $O_2^{\bullet -}$ production by titanium dioxide (TiO_2)-based sono-photocatalytic (SPC) system.[17] Recently, a variety of ultraviolet (UV)-driven photochemically active catalysts designated as photocatalysts consisting of TiO_2 has been developed[18-21] and applied for hygiene and antimicrobial purposes.[22,23] The likely mechanism of such catalysts involves the generation of ROS on the surface of the catalysts as expected (not fully proven) from the previously proposed models.[24] However, no attempt to confer long-lasting chemical properties to the waters (e.g., preparation of waters rich in ROS) has been reported, except for our model, despite of the increasing demands for the use of photocatalysts in various environments including the use in aqueous phase.

Our attempts were firstly, to obtain the data scoring the rate of $O_2^{\bullet -}$ production by both sono- and photo-catalytic manner, and secondly, to testify if the sono-photochemical priming of oxygen saturated water results in continuous release of $O_2^{\bullet -}$ in the system lasting at least for a half hour or not. For above purposes, a novel water conditioning SPC apparatus (**Figs. 1A and B**) equipped with sheets of TiO_2-coated photocatalytic fibers were applied for the preparation of ROS-containing water.[17] The apparatuses used in our demonstration was equipped with TiO_2-coated fibers and UV-A (360 nm) bulbs enabling the excitation of TiO_2, and also with 2 ultrasonic wave (USW) generating devices to assist the reaction of interest. When required, O_2 gas was supplied to the system through artificial lung (thus minimizing the impacts of air bubbles) connected with a magnet/roller pump. Monitoring of the dissolved O_2 level was necessarily required for enabling the optimal generation of $O_2^{\bullet -}$ in the water (5 L) maintained at *ca*. 20°C and circulated at 20 L/min.

In the model experiments, we detected the generation of hydroxyl radicals ($HO^{\bullet}$) (**Fig. 1C**) and $O_2^{\bullet -}$ (**Fig. 1D**) as the key members of ROS generated in the water circulated in SPC chambers, through electron spin resonance (ESR) spectroscopy and chemiluminescence (CL) assays, respectively. For CL assays, $O_2^{\bullet -}$-specific CL probe *Cypridina* luciferin analog (2-Methyl-6-phenil-3,7-dihydromidazo[1,2-a]pyrazin-3-one; designated as CLA;[25] was used. For ESR, a spin trapping agent, DMPO (5, 5-Dimethyl-1-pyrroline-*N*-Oxide) that readily forms an adduct

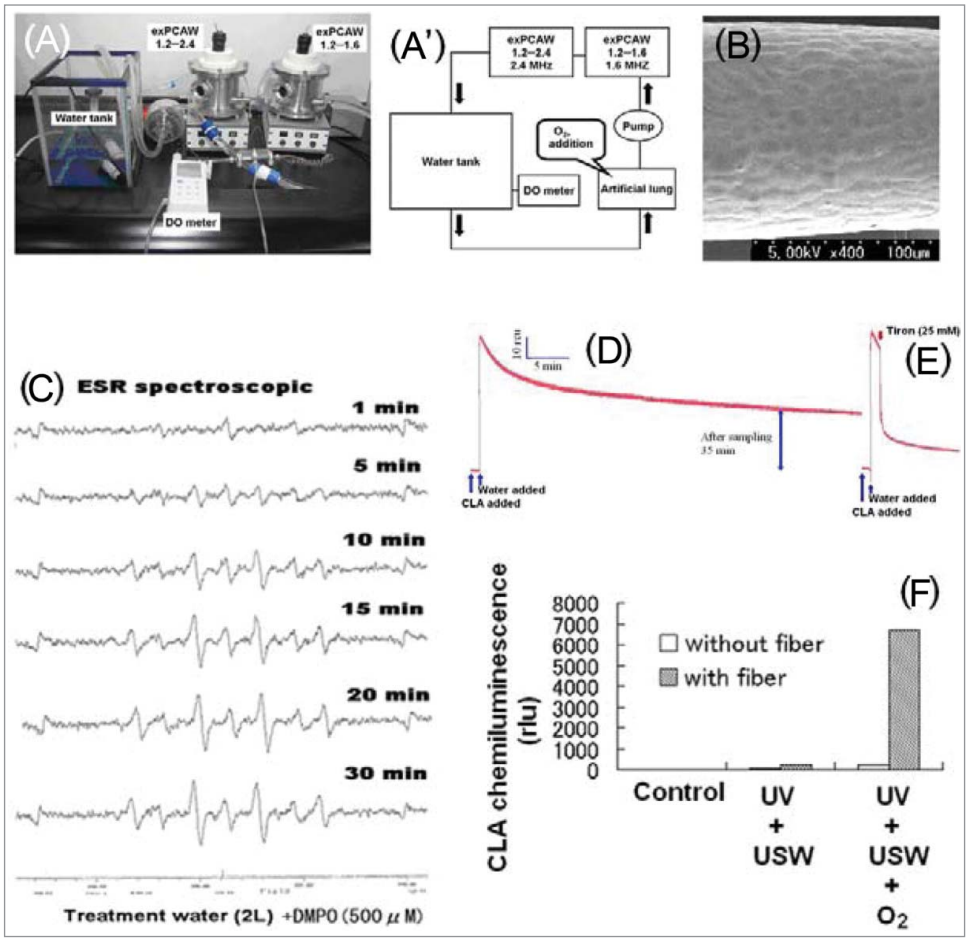

Figure 1. Generation of ROS by SPC processes. (a, a') Water conditioning SPC apparatus. Two exPCAW1.2s (WIPO No: WO10/032765; K2R Inc., Kitakyushu, Japan) connected in tandem (**A**) and the diagram of water conditioning system (a'). (**B**) Scanning electron microscopic image of the catalytic fiber which was fabricated by finely coating TiO_2 over alumina layer of aluminum fibers. (**C**) Detection of DMPO-OH signal with ESR reflecting the generation of $HO^{\bullet}$. (**D**) Monitoring of long lasting redox activity in the SPC-processed water using $O_2^{\bullet -}$-specific CL probe, CLA. (**E**) Quenching of CLA-CL by Tiron, a scavenger of $O_2^{\bullet -}$. (**F**) Synergistic impact of UV and USW in generation of $O_2^{\bullet -}$. Images were obtained and modified from.[17]

with $HO^{\bullet}$ was used. Note that CL reflecting the generation of $O_2^{\bullet -}$ can be detected in the water transferred out of the water circulating system, suggesting that some intermediate species or precursors of $O_2^{\bullet -}$ can be produced for certain length of time (**Fig. 1D**). In contrast, $HO^{\bullet}$ was detected only when the trapping agent, DMPO was circulated inside the SPC chambers. DMPO added to the water manually sampled out of the apparatus did not provide the signal for $HO^{\bullet}$ (data not shown).

One likely use of the conditioned water is controlling of the biological responses of living plant cells since it has been well documented that various physiological and biochemical events during the plant life cycle, such as germination of seeds, induction of defense mechanism against pathogenic microorganisms and adaptation to severe environments, are controlled by ROS.[24] It has been shown that expression of a number of redox-induced stress-related genes is regulated through calcium signaling in plant cells.[8] Therefore, pre-treatments of plants with redox-modifying agents that target the calcium channel opening may result in induction of anti-oxidative capacity (conferred by expression of ROS-responsive genes).

We proposed an attempt for conferring anti-oxidative capacities to plants for protesting the damaging impacts of photochemical oxidants or other oxidative stress, by novel water-processing technology which provides redox-active water designed for stimulating the expression of redox-related and defense-related genes in plants, through pre-treatments with moderate oxidative stresses.

To assess if the level of ROS produced in the photochemically conditioned water attained and remained at the level actively inducing the responses of living plant cells, we tested the responses of tobacco cell suspension culture (BY-2, expressing aequorin gene) to SPC-conditioned water. Presence of $O_2^{\cdot -}$ in the conditioned water-treated cell suspension culture was detected with the CL of CLA (**Fig. 2A**). Similarly, addition of SPC-conditioned water to tobacco BY-2 cells expressing aequorin gene resulted in increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (**Figs. 2B and C**) and slight increase in cell death (**Fig. 2D**). Similarly, we have shown that addition of nano-sized particles of TiO_2 induces the generation of ROS and also an increase in $[Ca^{2+}]_c$ in living tobacco cells.[26]

In addition, we observed that treatment of plants of ROS-sensitive tobacco cultivar (Bel-W3) with the SPC-conditioned water resulted in induced expression of PR1a, a ROS-responsive gene known to be involved in pathogenesis response in plants (**Fig. 2E**). Our proposal to use this novel water-treatment technology based on SPC has potential for vaccinating the plants, thus confering the resistance to oxidative stresses as illustrated in **Figure 2F**.

Learning from the chemico-biological models in plants (1): plant peroxidase as model enzyme

Living plants are sources of a number of enzymes involved in metabolism (production and removal) of ROS.[7] In higher plants, 2 major mechanisms are known to be responsible for production of ROS, namely one involving NADPH oxidases[27] and one involving peroxidases.[28] A research group in Geneva University metaphorically described that plant enzymes belonging to

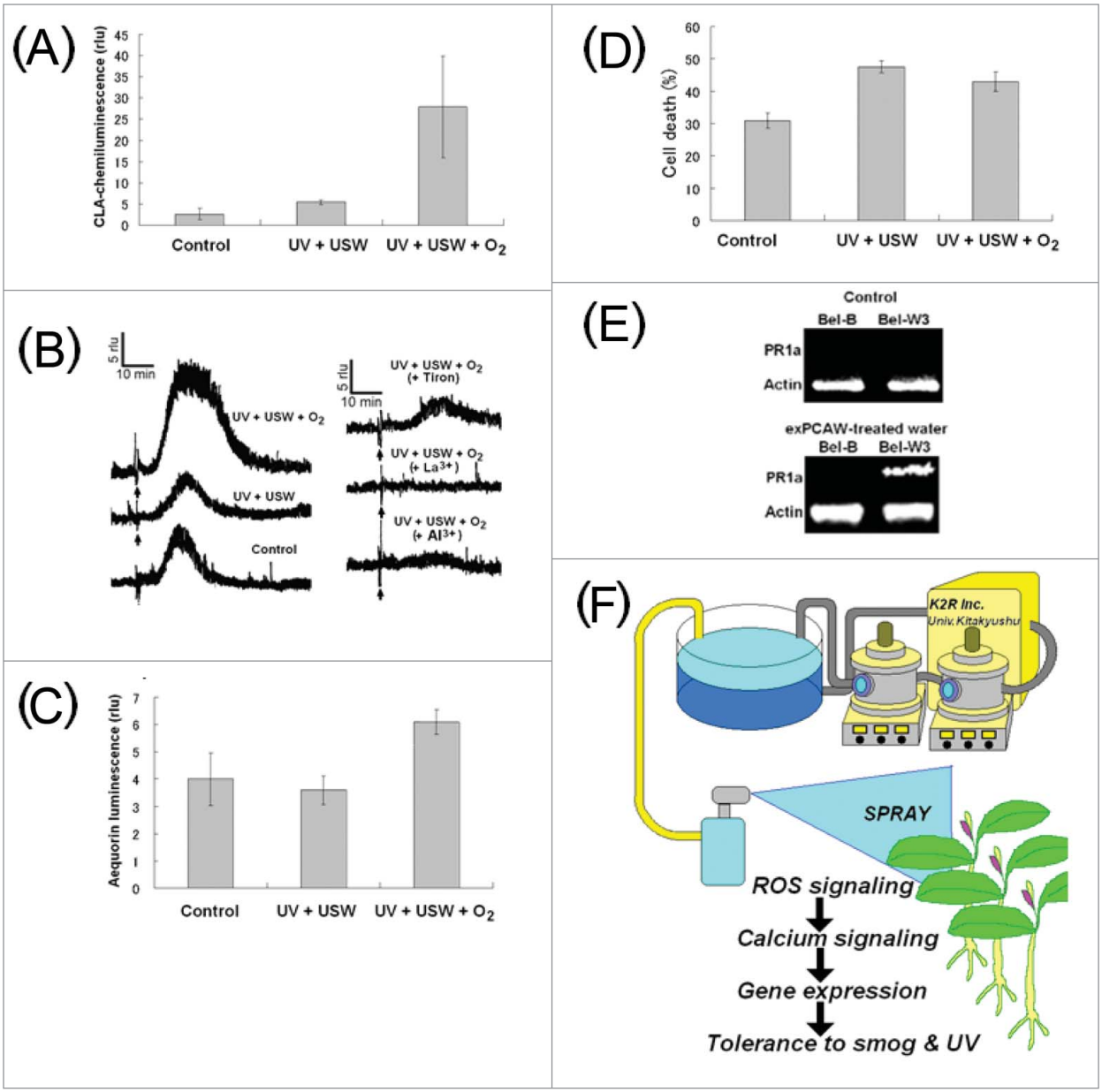

Figure 2. Biological impact of SPC-treated water. Effect of SPC-processed waters on $O_2^{\bullet -}$ production (**A**), induction of calcium influx into tobacco cells measured with aequorin luminescence (**B and C**) and cell death assessed with Evans Blue staining (**D**). (**E**) Induction of a ROS-responsive gene (PR-gene) expression by SPC-treated water. (**F**) Proposed use of SPC-treated water for hardening of plants eventually conferring stress tolerance to living plants. Effects of UV, USW and oxygen were compared in (**A–D**). Arrows in (**B**) indicate the timing of processed water addition. Data on gene expression analysis is cited from,[106] (oral presentation).

peroxidases ($_{EC}$ 1.11.1.7) display multiple functions more than a 'Swiss army knife'.[29] In the conventional peroxidase cycle, hydrogen peroxide (H_2O_2) is used as the common acceptor of electrons (e^-), and a variety of compounds function as e^--donating substrates.[28] Therefore, regulation of the level of H_2O_2 and oxidation of a wide range of substrates are the likely functions of plant peroxidases. Furthermore, diversified roles for plant peroxidases also include concomitant generation of ROS coupled to oxidation of phenolics such as salicylates,[8,30] and aromatic amines such as phenylethylamine.[31,32] As above, it is now accepted that plant peroxidases are capable of catalysis leading to generation of ROS, chiefly $O_2^{\bullet -}$, through oxidation of key substrates through H_2O_2-dependent conventional peroxidase cycle involving the cyclic formation of redox active enzyme intermediates known as Compounds I and II (path 3→5→4→3 in **Fig. 3**).[7,8,28] Generation of $O_2^{\bullet -}$ *via* peroxidase cycle involves the H_2O_2-dependent generation of intermediate radical species such as phenoxyl radicals or aromatic amine radicals which could be detected by ESR spectroscopy by using ascorbic acid as a spin trapper.[31-34]

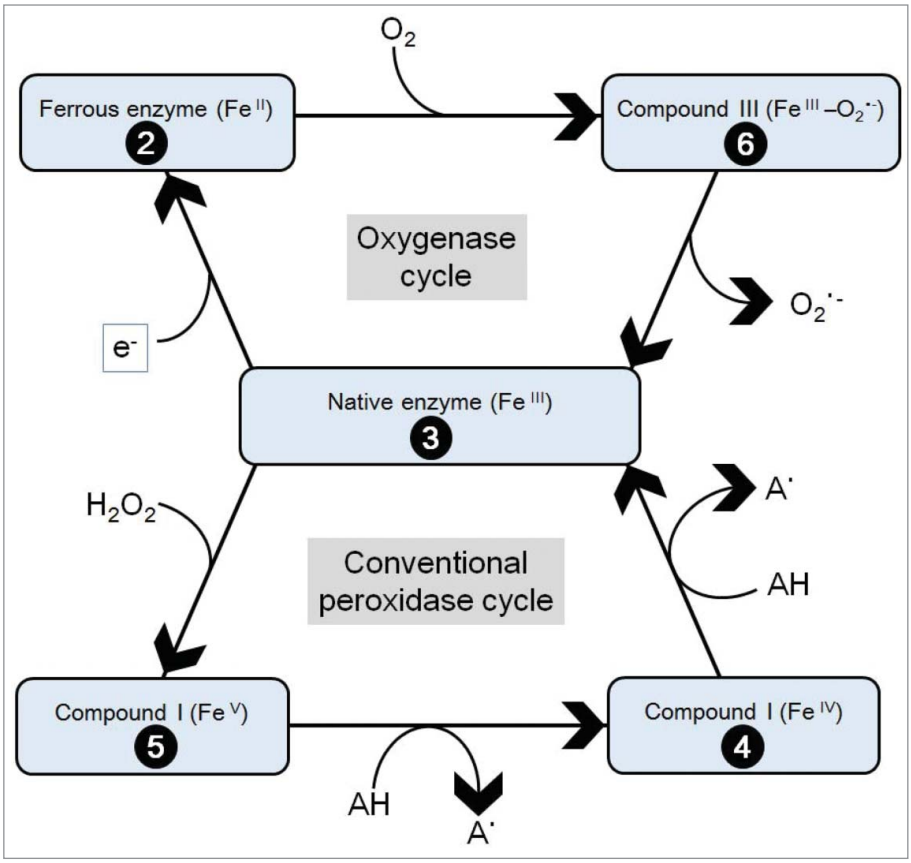

Figure 3. Hourglass model that summarizes the inter-conversions among active and inactive forms of peroxidase intermediates. The model was simplified based on earlier works.[28,43,106] The numbers on the black balls indicate the formal oxidation states of enzyme and its intermediates. Formation of radical intermediate ($A^{\bullet}$) further participates in formation of $O_2^{\bullet-}$. This model dissecting 2 distinct cycles initiated by interaction of native POX with acceptors and donors of e^-, is often referred to as the hourglass model due to its shape.[8,28,106]

In addition to aforementioned e^- acceptor-driven (H_2O_2-requiring) conventional peroxidative cycle, there is an alternative mechanism for generation of $O_2^{\bullet-}$ in an e^- donor-dependently driven manner, which should be described as the oxygenation or oxygenase cycle of plant peroxidase (path 3→2→6→3 in **Fig. 3**). The most widely known substrate for this H_2O_2-independent cycle is indole-3-acetic acid (IAA), the principal form of natural auxin in higher plants.[35-38]

We view that the role of IAA in POX-catalyzed generation of $O_2^{\bullet-}$ is one of effective e^- donors converting native enzyme into ferrous intermediate in the oxygenation cycle (**Fig. 3**, upper half). IAA-dependent reduction of native enzyme (with heme at Fe^{III}) into ferrous intermediate (with heme at Fe^{II}) is immediately followed by a series of reaction proceeding under the atmospheric O_2-rich condition, by which, unstable ferrous complex can be readily converted to O_2-bound form of enzyme intermediate known as Compound III in which the state of heme iron can be described as O_2-heme-Fe^{II} or $O_2^{\bullet-}$-heme-Fe^{III}.[39] Then, gradual decay of this complex into native enzyme at heme-Fe^{III} state accompanies the release of $O_2^{\bullet-}$ (**Fig. 3**, upper half) as confirmed with IAA-stimulated horseradish peroxidase (HRP) using $O_2^{\bullet-}$-specific CL probe, CLA.[37]

Interestingly, medical application of HRP-labeled antibodies and IAA, novel $O_2^{\bullet-}$-generating agents enabling cancer-targeted cell death induction, has been proposed based on the views that transient formation of [POX-IAA-O_2] complex results in robust release of $O_2^{\bullet-}$.[37] To date, 3 approaches have been reported, namely, HRP-conjugated immuno-labeling of cancer-related molecules,[4,40] expression of recombinant HRP in mammalian cells,[41] and modulation of the IAA-induced reaction using a fungal molecule.[38]

Assuming that the hypothetical model mechanism proposed in **Figure 3** (upper half) is correct, we should be able to screen or identify some effective e^- donors from a variety of single e^- reducing agents which target the native enzyme to trigger the onset of oxygenation cycle in plant POXs, eventually leading to a robust and long-lasting burst of $O_2^{\bullet-}$ production. After testing a wide range of chemicals, we observed that free ferrous ion (Fe^{2+}) acts as a novel inducer of $O_2^{\bullet-}$ production in aid of plant POX, possibly by behaving as an effective e^- donor for Fe^{III}-to-Fe^{II} conversion of heme in a model POX, HRP. The aim of the present section is to share our novel finding on the Fe-driven $O_2^{\bullet-}$ production mechanism involving HRP.

In plants, physiological significance of such peroxidase-mediated redox reaction could be found in the defense mechanism against microbial attacks. Recently, we have proposed a likely role for non-biological inorganic factors such as free ferrous ion[42] and nitric oxide[43] in reduction of ferric native enzyme into ferrous intermediate protein which readily produces $O_2^{\bullet-}$ through the mechanism involving the formation and decay of Compound III (Path 3→2→6→3 in **Fig. 3**, upper half), thus, possibly contributing to the plant defense mechanism. By making use of the oxygenase cycle in plant peroxidase, modulation of the enzyme activities by doping-chemicals without supplementation of any substrate is one of the artificial regulatory modes of peroxidase.

Learning from the chemico-biological models in plants (2): Plant-derived short peptides H_2O_2-dependently producing $O_2^{\bullet-}$

Ozone (O_3) is a major secondary air pollutant, threatening the living plants, often reaching high concentrations in the urban areas under intensive solar ray. It is generally understood that generation and/or removal of ROS form the key signaling events governing the behaviors of living plants at cellular level under the

O_3 stresses.[44,45] Interestingly, one of key factors required for plant survival under O_3 toxicity was shown to be endogenous peroxidase[46] since the level of ROS determines the onset of localized apoptotic cell death in O_3-exposed cells.[47,48]

In addition to plant peroxidases, small peptides behaving similarly to peroxidases reportedly participate in the plant responses to O_3.[9] As one of O_3-induced plant responses, expression of O_3-inducible (OI) genes have been identified in saltbush (*Atriplex canescens*) and few other plants.[49] OI gene inducers include some additional chemical and environmental factors such as SO_2 and water deficit, suggesting the multiple roles for these genes.[49] By No and his colleagues,[50] 2 isotypes of OI peptides, OI2–2 (158 amino acids) and OI14–3 (119 amino acids), were identified. These peptides possess a common repeat unit (8–10 times repeated in tandem), consisting of hexa-amino acids (Y-G-H-G-G-G). OI peptides are considered to be putative members of glycine-rich proteins designated as GRPs, due to the presence of above repeat unit.[50] The secondary structures of most GPRs and OI peptides are rich in β-pleated sheets,[49-51] suggests that there is some similarity between prion proteins (PrPs) from animal systems and plant GRPs including OI peptides.

Recently,[51] it was suggested that the hexa-repeat in OI peptides acts as a metal-binding motif and synthesized model peptides with the hexa-repeat found in OI peptides are highly active in generation of $O_2^{\bullet-}$, upon addition of H_2O_2 to the copper-loaded peptides. Interestingly, in the above report, possible mechanism of the reaction and biological consequence of the reactions catalyzed by OI peptides were discussed by analogy to the action of octarepeat peptides derived from human prion protein (PrP) as discussed later.

Role of His and Tyr residues in redox-active peptides

As described above, plant-derived OI peptides containing PrP-like repeated sequence consisted of a His- and Tyr-containing hexa-repeat unit repeated for 8–10 times in tandem.[50] Accordingly,[51] the repeat unit found in the O_3-induced peptides shows H_2O_2-dependent $O_2^{\bullet-}$ producing activity by using self-Tyr residues as phonolic substrates required for peroxidative process, thus simply converting H_2O_2 to $O_2^{\bullet-}$ without requirement for additional phenolics or amine as substrates. As mentioned above, 2 OI peptides, OI2–2 and OI14–3, share the identical repeat motif (G-G-G-Y-G-H) which is repeated for 9 and 7 times, respectively. The each repeat unit in OI peptide was shown to form a complex with transition metals, chiefly copper ion in the physiological pH range based on the spectroscopic studies by measuring the cyclic dichroism (CD) and nuclear magnetic resonance (NMR).[52] Yokawa et al. have assessed the $O_2^{\bullet-}$-generating activity of metal-binding motif derived from plant OI peptides, by chemically synthesising a series of peptides, namely G-G-G-Y-G-H, Y-G-H-G-G-G, H-G-G-G-Y-G, and G-G-G-F-G-H.[51] To prevent the peptide from forming self circularization, amino and carboxyl terminal of each peptide was acetylated and amidated, respectively.

Accordingly, the peptide with His residue located at the carboxyl terminal (G-G-G-Y-G-H) showed the highest activity and that with N-terminal His residue (H-G-G-G-Y-G) showed no activity. Therefore, not only the presence, but also the location of His residue is one of critical requirement for the reaction.[51] Similar observation confirming the role for His residue have been reported for the model His-containing Cu-binding peptides as discussed in the below sections of this article.[53]

By analogy to the putative role for Tyr residue as intramolecular substrates within some redox active enzymes such as cyclooxygenase-2[54] and ribonucleotide reductases,[55] in which corresponding reactions proceed *via* the formation of a tyrosyl radical, the importance of Tyr residue in the repeat unit in OI peptides was assessed. Accordingly, a Tyr-to-Phe (Y-to-F) substituted mutant peptide (G-G-G-F-G-H) was synthesized and used for comparison. As predicted, the Cu/G-G-G-F-G-H complex showed no activity for the generation of $O_2^{\bullet-}$ after the addition of H_2O_2.[51] On the other hand, by supplementation of free Tyr as a substrate into the reaction mixture containing Cu/G-G-G-F-G-H complex, the H_2O_2-dependent $O_2^{\bullet-}$-generating activity was regained. Requirement for free Tyr or Tyr residue in H_2O_2-dependent $O_2^{\bullet-}$-generating reaction suggest that tyrosyl radical (one form of phenoxy radicals) is transiently formed for one-electron reduction of O_2 similarly to the plant peroxidase reaction H_2O_2-dependently generating $O_2^{\bullet-}$ by coupling to oxidation of phenolics (such as salicylic acid) to form phenoxy radicals.[33,56]

Finding and defining the metal-binding and catalytic motifs within chicken prion proteins

There is a set of proteins which could not be defined by proposition (3)

$$\{Es\} \in \{\{Cs\} \cap \{\text{proteins} \cup \text{peptides}\}\}$$

The case of prion proteins (PrPs) and derived small peptides could be one such example. Generally, PrPs and derived peptides are not considered as enzymes at present, although they are either proteins or peptides having catalytic nature (proposition 11).

$$\{PrPs\} \in \left\{\left\{\{Cs\} \bigcap \left\{\text{proteins} \bigcup \text{peptides}\right\}\right\} \setminus \{Es\}\right\} \quad (11)$$

By admitting that there are proteins or peptides (both natural and artificial) with catalytic activity which can be considered as element of {*BCs*} in a broad sense as defined below (proposition 12), the phenomena observed with plant OI-peptides and animal PrPs belonging to novel class of *BCs* can be compared with conventional *BCs* such as plant peroxidases.[9]

$$P(\text{novel}BCs) = \exists \left\{\text{proteins} \bigcup \text{peptides}\right\} \in \left\{\{BCs\} \setminus \{Es \cup Ns\}\right\} \quad (12)$$

Actually, the kingdoms of plants and animals are rich in such small peptidic metalloenzymes, belonging to *BCs* in a broad sense, catalyzing the generation of $O_2^{\bullet-}$.[53]

The criteria for consisting a minimal peroxidase-like small peptides is the presence of His-rich motifs required for binding to metals (chiefly copper), and free and/or peptide-bound substrates.[51] Similarly, recent studies have shown that peptides

derived from human PrP mediates the production of $O_2^{\bullet -}$ through oxidation of substrates such as aromatic monoamines or phenolics (mostly, neurotransmitters and their analogs).[57] Upon binding to copper at 4 different putative copper-binding motifs (**Fig. 4A**), PrP and derived peptides may gain the catalytic activities as our earlier works have revealed that PrP-derived copper-binding peptides catalyze the generation of $O_2^{\bullet -}$ in peroxidase-like manner involving H_2O_2 as e^- acceptor and aromatic amines or phenols as the e^- donors.[57,58]

Actions of Cu-bound PrPs are of great importance from the engineering point of view in order to design the novel peptidic metalloenzymes. Apart from engineering purpose, but viewing from the biological and medical points, the importance of metalloproteins in neurobiology has been suggested both as oxidants and antioxidants in neurodegenerative processes in animals.[59] Cu is an essential trace element in most living organisms but its redox reactivity often leads to the risk of oxidative damage to the cells and tissues, as observed in the neurodegenerative diseases such as 'prion' disease and Alzheimer, Menkes' and Wilson's diseases all occurring *via* disorders of Cu metabolism.[60–62] Especially, Alzheimer disease and prion disease are 2 of known major conformational diseases, as documented to date.

Deposition of abnormal protein fibrils is a common pathological feature observed in "protein conformational" diseases, including prion dementias and Alzheimer, Parkinson and motor neuron diseases.[63] Generation of ROS is now considered as one of key events required for development of conformational diseases. In the cases of accumulation of α-synuclein in Parkinson disease and accumulation of β-amyloid in Alzheimer disease, the evidence for involvement of ROS, chiefly H_2O_2 and derived $HO^{\bullet}$, in the neurodegenerative mechanisms have been documented, suggesting that pathogenesis of such neurodegenerative diseases could be attributed to the generation and damaging impacts of ROS which eventually stimulates the formation of abnormal protein aggregates.[63,64]

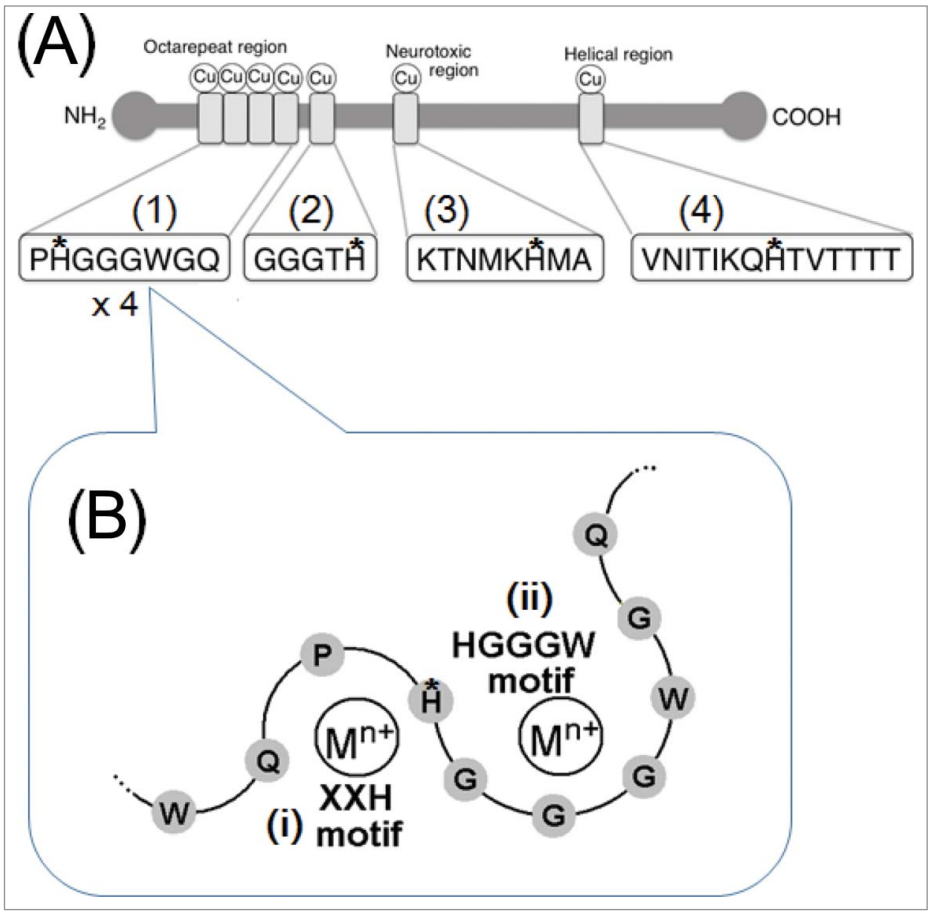

Figure 4. Copper-binding motifs in human PrP. (**A**) Putative copper-binding domains present in human PrP. Four peptide sequences corresponding to copper-binding motifs in PrP. (1) The most characterized Cu-binding sequence repeated 6 times in bovine and 4 times in human, mouse and ovine. (2) An additional Cu binding sequence found in the basic region in human and mouse PrPs immediately after the octarepeats. (3) PrPs from mammals and chicken show similar sequences. (4) Well characterized PrP helix 2 sequence.[57] (**B**) Recently proposed overlapping motifs in the human PrP's octarepeat region required for binding to metals. (i) X-X-H motif[9,76,77] suggested to bind Tb in the Tb-fluorescence assay. (ii) H-G-G-G-W motif[80] suggested to bind Cu in the peptide-fluorescence assay. M^{2+}, metal cations. Histidine residues possibly involved in anchoring of copper are marked with asterisks.

PrPs are the only known causative agents for transmissible spongiform encephalopathies in mammalian brains.[65] A number of studies have shown that PrPs can form a group of Cu-binding proteins possibly involved in redox reactions[66,67] as human PrP has 4 Cu-binding sites in the "octarepeats" region (PrP 60–91) in which amino acid sequence P-H-G-G-G-W-G-Q appears 4 times in tandem and each repeat possibly binds single Cu^{2+} at physiological neutral and basic range of pH.[68] Similarly, in chicken PrP, the Cu-binding motif analogous to the octarepeats are known as hexa-repeats in which each repeat consist of the 6 amino acids, H-N-P-G-Y-P. In chicken PrP, His residues in hexa-repeat are considered to play a key role in anchoring of Cu.[69]

Note that both His and Tyr residues can be found in the chicken PrP's hexa-repeat unit. As Tyr-containing peptides could be a target of the redox reaction catalyzed by metal-containing proteins or peptides involved in peroxidative and ROS generating reactions, we synthesized 6 peptides corresponding to Cu-binding region (hexa-repeat) of chicken PrP and examined its catalytic activity for the generation of $O_2^{\bullet -}$. Each of 6 peptides synthesized (N-P-G-Y-P-H, P-G-Y-P-H-N, G-Y-P-H-N-P, Y-P-H-N-P-G, P-H-N-P-G-Y, and H-N-P-G-Y-

P) contains both His residue possibly anchoring Cu ion and Tyr residue possibly behaving as a substrate were used for assessing the $O_2^{\bullet-}$generating activity using the $O_2^{\bullet-}$-specific CL of CLA.[70] As a results, the generation of $O_2^{\bullet-}$ was observed in the presence of hexapeptide, copper and H_2O_2 without addition of any phenolic substrate since Tyr-residue on the hexapeptide possibly behaves as a substrate for the reaction.

To conclude that requirement of Tyr residue on the peptides in H_2O_2-dependent generation of $O_2^{\bullet-}$, we also tested the mutation of Tyr resides into Phe residues in 2 model peptides (*i.e.* N-P-G-F-P-H and F-P-H-N-P-G). As expected, the Y-to-F substitution mutant peptides showed complete loss of H_2O_2-dependent generation of $O_2^{\bullet-}$. Furthermore, we confirmed that supplementation of free Tyr to the reaction mixture containing the Y-to-F mutant peptides results in production of $O_2^{\bullet-}$. It is conclusive that similarly to plant OI-peptide, both the presence and positions of His and Tyr residues in chicken PrP's hexarepeat units are highly important for the catalytic modes leading to generation of $O_2^{\bullet-}$.

Catalytic activity in human prion-derived peptides

To date, key involvement of trace elements, chiefly of Cu, in prion disease has been well documented.[71,73] Until recently, 2 opposing roles for Cu-bound PrPs have been proposed and discussed, namely the role of bound copper as an anti-oxidant element and contrary as a pro-oxidant element enhancing the neurodegenerative process.[74] In both cases, Cu-binding sequences highly preserved in PrPs play key roles in generation[57] or removal of ROS.[72]

A series of works conducted by our group suggested that 4 distinct peptide sequences corresponding to 7 putative copper-binding sites containing metal anchoring His residues (His61, His69, His77, His85, His96, His111, and His187) in human PrP function as catalytic motifs active for $O_2^{\bullet-}$ generation through reactions with aromatic monoamines.[57] Furthermore, phenol-dependent $O_2^{\bullet-}$ generation catalyzed by several PrP-derived copper-binding peptides was recently assessed using various phenolics as substrates such as free phenolics, free Tyr,[53,75] solubilized polymers with phenolic groups (*i.e.,* polyvinyl phenol which is a polymer with multiple phenolic groups, chain length, 12–58 mer,[9] and Tyr residues on peptide chains.[58] Since supplementation of H_2O_2 is required for oxidation of amines or phenols by these copper-centered peptides, the modes of reactions were considered to be peroxidase-like.[9,57]

Among PrP-derived and related Cu-binding motifs ever examined, H_2O_2-dependent $O_2^{\bullet-}$-generating activity was most active in a truncated helical sequence (V-N-I-T-K-Q-H-T-V-T-T-T-T) which is highly analogous to original (wild-type) PrPs' helical sequence.[57,75]

Two distinct metal-binding motifs overlaid in the PrP octarepeat region

Our earlier studies have revealed that His residues (at least single His) are required for anchoring copper on PrP-derived peptides,[57,76] and consequently, the catalytically active copper-binding motif within PrP-derived peptides was determined to be X-X-H, where X can be any amino acids followed by His residue.[9,77]

In human PrP, His96 is located between G-G-G-T and S-Q-W-N sequences. To examine the positional effect of His on the catalytic activity in the derived peptides, comparison of the His-started H-S-Q-W-N pentapeptide and the His-ended G-G-G-T-H pentapeptide was carried out.[53] While reaction with tyramine (given as model substrate) and G-G-G-T-H peptide resulted in robust production of $O_2^{\bullet-}$, the H-S-Q-W-N peptide showed no catalytic activity. By assuming that G-T-H motif within the G-G-G-T-H pentapeptide is one of X-X-H motif derivatives, experimental comparison of the catalytic activities among G-G-G-T-H pentapeptide and shorter derivatives (G-G-T-H and G-T-H) were performed and the data obtained clearly suggested the importance of the *N*-terminal glycyl-chain elongation for manifesting the maximal redox activity in *C*-terminal His anchored peptides.

Example of artificial enzyme based on XXH motif was developed,[78] demonstrating that Cu-binding peptides with X-X-H motif conjugated to organic materials could form a novel class of biosensing and bioengineering tools. Accordingly, a tripeptide, Gly-Gly-His (G-G-H, one of X-X-H motif derivatives) was introduced onto the glycidyl methacrylate-grafted porous hollow fiber membrane made on the polyethylene platform by radiation-induced graft polymerization. After loading of Cu^{2+} on the membrane, CL assay was performed to testify the catalytic activity of the membrane, generating $O_2^{\bullet-}$ upon addition of H_2O_2 and tyramine as the paired substrates. Inokuchi et al.[79] have studied the minimal motifs required for binding of metals within human PrP, by assessing (1) the peptide-dependent quenching of Tb^{3+} fluorescence and (2) the Cu^{2+}-dependent quenching of intrinsic fluorescence in human PrP octarepeat-derived peptides. Nobel assays based on the quenching of Tb-fluorescence by interacting peptides emphasized the role of His-ended peptides sharing X-X-H motif. The obtained data clearly supported the view that an intact X-X-H motif located at *C*-termini of peptides, is desirable as the site of metal chelation. In the case of human PrP's octarepeat unit, P-Q-H motif rather than *N*-terminal H-G-G-G-W motif was shown to be active in metal binding.

Empirically, *N*-terminal His-started oligo-peptides derived from human PrP have been used as models for Cu-binding in earlier *in vitro* studies. These study suggested that the actual least motif in the octarepeats necessarily required for binding of Cu consists of 5 amino acids H-G-G-G-W[80] or 4 amino acids H-G-G-G.[68]

Interestingly, the role of His-started motif (H-G-G-G-W) was supported by the Cu-dependent peptide fluorescence quenching assay.[79] Among the octapeptide sequences examined, the P-H-G-G-G-W-G-Q peptide was shown to be the most sensitive to the low Cu concentration although this sequence lacks the presence of intact metal-binding X-X-H motif.

Taken together, in the mammalian PrP octarepeat regions, in which P-H-G-G-G-W-G-Q is repeated for 4 (human) to 6 (bovine) times, 2 distinct metal binding motifs, namely, X-X-H motif and H-G-G-G motif, could be overlaid by sharing common His residue and thus co-existed (**Fig. 4**).

Role of Tyr residues within human PrP, the likely targets of catalysis

Human PrP-derived catalytic model peptides all showed requirements for addition of aromatic substrates in order to produce $O_2^{\bullet-}$ in the presence of H_2O_2[53,57] while the studies with the Cu-binging motifs in plant OI-peptides and chicken PrP are strongly indicating that Tyr residues presented on the peptide chains are the likely target of redox relay eventually converting H_2O_2 into $O_2^{\bullet-}$.[9,70] Since free Tyr (among the active phenolics examined) is a good substrates for human PrP-derived catalytic short peptides, we assume that the modes of catalytic actions among the plant-derived, chicken-derived and human-derived copper-binding peptides described above may not differ much.

In case of non-peptidic free Tyr given as a model substrate, the presence of phenolic moiety, but not the amino and carboxyl groups, was shown to be important in the interaction with Cu-bound PrP-derived peptide.[58] Therefore, it is tempting to testify if the Tyr residues flanking on peptidic chains or proteins function as putative targets of human PrP's copper-binding motifs.

In fact, human PrP possesses several Tyr-resides being exposed to the external media and events involving such Tyr residue may play a pivotal role in development of prion dementias, as recent reports suggested that helix H1 of human PrP and its 2 flanking loops (highly rich in Tyr residues) are subjected a transition into a β sheet-like structure during forced conformational conversion of the intrinsic cellular form of PrP (PrP^{c}) into the scrapie form of PrP (PrP^{sc}) (Bertho et al., 2008). By definition, conversion of the PrP^{C} into PrP^{SC} is a fundamental event observable upon onset of prion disease development. Yokawa et al.[58] have reported an attempt to testify if the Tyr residues on PrP or derived peptides can be used as the substrate for a human PrP-derived Cu-bound catalytic peptide. In their experiments, the Cu-bound V-N-I-T-K-Q-H-T-V-T-T-T-T helical peptide was used as a model catalyst H_2O_2-dependently producing $O_2^{\bullet-}$. On the other hand, the tested putative substrates include (1) tyrosyl-tyrosyL-arginine tripeptide (Y-Y-R) which appears twice in the PrP's Tyr-rich region (DYEDR-YYR-ENMHRYPNQV-YYR-PMDEY) and (2) longer peptide sequences corresponding to the Tyr-rich region in human PrP (D-Y-E-D-R-Y-Y-R-E-N-M-H-R).

Compared to free Tyr, Y-Y-R tripeptide was shown to be much more active in production of $O_2^{\bullet-}$, confirming that both free form and peptide-integrated forms of Tyr can be recognized by the Cu-loaded catalytic peptide.[58] Although the reactivity of longer peptide sequences corresponding to the Tyr-rich region in human PrP (D-Y-E-D-R-Y-Y-R-E-N-M-H-R) was obviously lower than free Tyr, comparison with the Y-to-F substitution mutant (D-F-E-D-R-F-F-R-E-N-M-H-R) confirmed that Tyr-rich long peptides are favored for production of $O_2^{\bullet-}$. These data suggest that the Tyr residues presenting on the intra- and inter-PrP molecules could be the target of the Cu-bound PrP-catalyzed reaction.

Synthesis of novel metalloenzymes with peptides and their substrate preferences

Among human PrP-related Cu-binding model peptides, the octarepeat unit (P-H-G-G-G-W-G-Q) was shown to be active in aromatic monoamine (AMA)-dependent $O_2^{\bullet-}$ generation using phenylethylamine as model substrate,[57] by mimicking the plant AMA-utilizing enzymes sensitive to monoamine oxidase inhibitors.[31] On the other hand, a helical motif V-N-I-T-K-Q-H-T-V-T-T-T-T-T undecapeptide and G-G-G-T-H pentapeptide, both derived from human PrP, showed negligible AMA-dependent activity while performing much greater phenol-dependent $O_2^{\bullet-}$ generating activities.[53,57,58] Based on above knowledge, substrate specificity of novel metalloenzymes can be properly designed. Based on the results with PrP-derived peptides, our group has designed a series of novel peroxidative biocatalysts as discussed below.

By analogy to G-G-G-T-H, a phenol-oxidizing catalytic pentapeptide derived from human PrP, we have designed a series of simplified model peptides (G_nH series peptides) which are composed of oligoglycyl chains ended with *C*-terminal His. To test the importance of the elongated *N*-terminal glycyl chain and anchoring His residue, both G_nH series peptides varied in *N*-terminal glycyl chain length ($n = 2, 3, 4, 5$ and 10) and oligoglycyl peptides lacking His (G_n series) were synthesized.

As expected, G_n series showed no catalytic activity since these sequence lack the motif for binding to catalytically important Cu^{2+}. Within the G_nH series, catalytic activity of the minimal Cu-binging motif (G_2H tripeptide) was hardly detected despite the Okobira model.[78] Probably, in the Okobira model, in addition to G-G-H sequence, the supporting chains of glycidyl methacrylate on which G-G-H is grafted may playing a role similarly to *N*-terminal elongating oligo-Gly chain. In G_nH series, G_3H tetrapeptide showed a detectable increase in production of $O_2^{\bullet-}$, confirming the importance of *N*-terminal Gly elongation. It can be generalized that the catalytic performance in G_nH series can be *ca*. 3-fold enhanced by single amino acid elongation (addition of *N*-terminal Gly residue, allowing elongation from G_2H to G_3H, G_3H to G_4H, and G_4H to G_5H). However, further elongation from G_5H hexapeptide to $G_{10}H$ undecapeptide resulted in only *ca*. 3-fold of enhancement suggesting that the requirement for the *N*-terminal elongation is nearly fulfilled. These data suggest that the presence of the *C*-terminal His is the primary requirement for catalytic performance, and *N*-terminal elongation contributes to the enhancement of the catalytic activity.

Although involvement of Cu and generation of ROS are analogous to tyrosinase which oxidizes Tyr and polyphenols with concomitant release of $O_2^{\bullet-}$,[59] the roles played by H_2O_2 are largely different in the G_nH series metalloenzymes. While H_2O_2 is often regarded as an inhibitor of the tyrosinase reaction,[81] the G_nH series metalloenzymes require the presence of H_2O_2 as co-substrate. On the other hand, plant peroxidases are shown to be active in generation of $O_2^{\bullet-}$ upon oxidation of various phenolics and monoamines in the presence of H_2O_2,[28,33] suggesting that the modes of reactions catalyzed by PrP-derived peptides and artificial G_nH series metalloenzymes are analogous to the modes of peroxidase reactions.

Among hydroxybenzoic acids (HBAs) and benzoic acid (BA), 2-HBA (salicylic acid) and BA were shown to be poor substrates for $O_2^{\bullet-}$-generating reactions catalyzed by G-G-G-T-H pentapeptide and G_nH series metalloenzymes.[53] In contrast, 3-HBA

and 4-HBA were shown to be good substrates, suggesting that the presence of phenolic moieties with *m*- or *p*-positioned OH group is required for generation of $O_2^{\bullet -}$, notably differed from the plant peroxidase which favors 2-HBA.[56] When dihydroxybenzoic acids (DHBAs) were used as model substrates, inutility of *o*-positioned OH group was also observed using 2,6-DHBA. However, PrP-derived peptides (G-G-G-T-H and V-N-I-T-K-Q-H-T-V-T-T-T-T), plant OI-peptides, and G_nH series metalloenzymes showed $O_2^{\bullet -}$ generating activity upon addition of other DHBAs with *o*-positioned OH (2,3-DHBA, 2,4-DHBA, and 2,5-DHBA), indicating that the presence of *o*-positioned OH does not interfere with the roles for active OH groups at *m*- and *p*-positions.[9,53,58]

Kinetic analysis performed with G_nH series metallopeptides

G_5H hexapeptide, one of G_nH series artificial enzyme was selected for kinetic analyses based on the $O_2^{\bullet -}$-specific CL with CLA.[53] By calibrating the yield of $O_2^{\bullet -}$ using KO_2 as standard, the rate of $O_2^{\bullet -}$ production proceeding in the presence of tyramine was assessed. With Lineweaver-Burk analysis, K_m and V_{max} for the G_5H hexapeptide-catalyzed production of $O_2^{\bullet -}$ in the presence of tyramine were determined to be 0.42 mM and 0.12 mmol / mg peptide / min, respectively.

Enhanced thermo-stability in novel metalloenzymes

It has been reported that prion-infected brain tissues or homogenates hardly lose their infectivity even after severe heat treatment[82] and repeated freezing and thawing.[83] Yokawa et al[84] have hypothesized that redox activities reflected by the generation of ROS in PrP-derived Cu-binding peptides may also show thermo-stability, therefore, PrP-derived peptide may strive through heating and/or repeated freezing and thawing cycles. It is noteworthy that tyramine-dependent $O_2^{\bullet -}$-generating activity found in the peptides corresponding to the Cu-binding motifs in human PrP (G-G-G-T-H, V-N-I-T-K-Q-H-T-V-T-T-T-T), showed extreme thermo-stability surviving under heat-incubation (90°C, 100 min), autoclaving, and repeated freezing/thawing cycles, despite most enzymes and proteins are sensitive to high temperature and repeated freezing.

Newly designed metallo-peptides, G_5H hexapeptide and $G_{10}H$ undecapeptide were also used for thermo-stability assessment.[53] When the peptides (G_5H and $G_{10}H$) were incubated in the absence of copper, any loss of the catalytic activity was observed even after thermal denaturing treatments. In contrast, Cu-bound form of G_5H hexapeptide showed some extent of the loss in catalytic activity (*ca. 20*%) after autoclaving (121°C, 20 min) but this peptide survived the 100 min of heating at 90°C and 10-time repeated freezing and thawing cycles.

Signal transduction-sensitive artificial enzyme

Protein phosphorylation is associated with most cell signaling and developmental processes in eukaryotes. Owing to the introduction of phosphate, a bulky and highly charged group, the phosphorylating events often results in a drastic changes in the properties of proteins (mostly enzymes), eventually modulating the activity of enzymes or protein-protein interaction properties.[85]

In order to design a novel (probably first) signal transduction-sensitive artificial enzyme, a chimeric bio-catalyst designated as $ErkG_5H$ (**Fig. 5A and B**) was constructed,[15] by fusing G_5H, a PrP-inspired metalloenzyme[53] with Tyr-containing Erk1/2 MAP kinase (MAPKK) substrate sequence (erk1/2 residues, 182–187 MAPKK phosphorylation sites) consisting of F-L-T-E-Y-V-A.[86] Using this molecule, Tyr residue-assisted conversion of H_2O_2 to $O_2^{\bullet -}$ was successfully achieved without supplementation of phenolic substrates (**Fig. 5C and D**), due to the presence of a single Tyr-residue vicinal to Cu-binding catalytic motif (G_5H).

Then, $ErkG_5H$, consisting of (i) Tyr-containing substrate mimic region and (ii) the catalytic region, was used to study the impact of amino acid phosphorylation, which could be the first demonstration of the phosphorylation-regulated artificial enzyme.[15] Within short peptide sequence F-L-T-E-Y-V-A-G-G-G-G-G-H, 2 amino acid residues, namely, Thr and Tyr residues, can be the sites of phosphorylation since the original sequence was derived from the phosphorylation domain within MAPKK.[86]

In order to examine the phosphorylation-sensitiveness in $ErkG_5H$ molecule, $O_2^{\bullet -}$-generating catalytic activities in non-phosphorylated and phosphorylated peptides (Thr-phosphorylated, Tyr-phosphorylated, and Thr and Tyr double phosphorylated) were compared.[15] Catalytic activity of $ErkG_5H$ was completely lost in the double phosphorylated sample (**Figs. 5C and D**). Even by single phosphorylation at Tyr residue alone, catalytic activity of $ErkG_5H$ was mostly lost. In contrast, single phosphorylation at Thr residue resulted in partial inhibition only. Positional impact of phosphorylation determining the catalytic activity is summarized in **Figure 5E**. This work provided the first implication that phosphorylation-controllable artificial enzyme can be synthesized.

Interaction between DNA and redox-active metals

It is well known that oxidative damage to genomic DNA is promoted in the presence of ROS such as $HO^{\bullet}$, which can be generated *via* Fenton-type or Harbor-Weiss-type reactions in the presence of the Cu and Fe ions.[87] It is also known that the metal-mediated oxidative damage to DNA is further enhanced by coexisting ascorbic acid or H_2O_2.[88] Such oxidative DNA fragmentation and subsequent chromosomal dysfunction may play key roles in apoptotic cell death mechanisms in mammalian cells.[89] Through the DNA-degrading reactions in the system containing Cu(II) + H_2O_2 or Cu(II) + ascorbate, the production of a large amount of $HO^{\bullet}$ at physiological pH condition *via* Harbor-Weiss-like reaction has been recorded, by monitoring the level of 8-hydroxyguanosine which is a reliable biomarker for $HO^{\bullet}$-dependent oxidative damage to guanosine residues on DNA.[88]

It is known that Cu^{2+} strongly binds to the guanosine and cytidine bases at physiological pH, eventually perturbing the A-T base pairs and disrupting the double-helical structure of DNA.[90] In addition, specific regions on DNA, so called Z-DNA structure-like micro-domains, show much higher affinity to binding of Cu^{2+}, especially at the base guanine.[91]

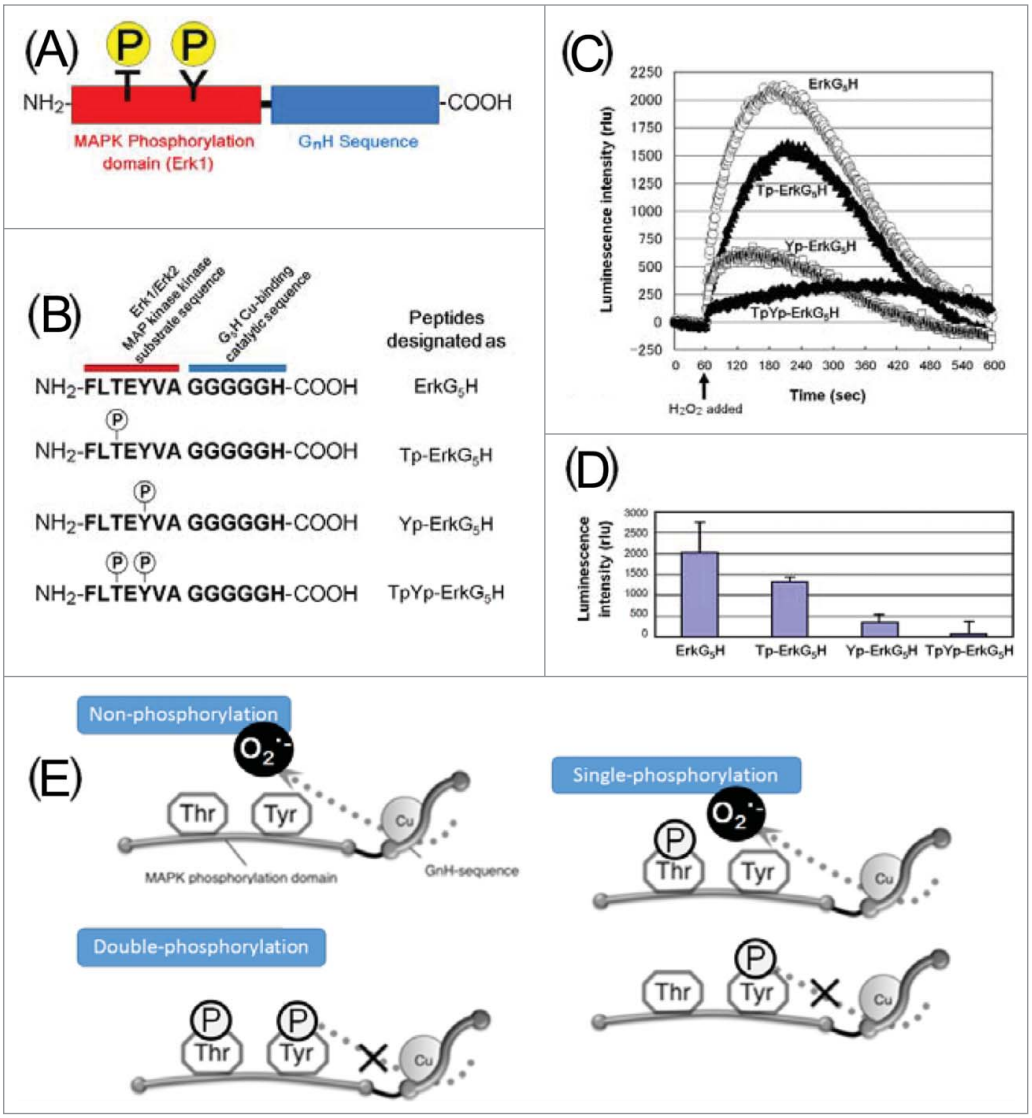

Figure 5. A novel phosphorylation-sensitive chimeric biocatalyst designated as $ErkG_5H$. (**A**) Proposed design of the novel phosphorylation-sensitive metalloenzyme. (**B**) Phosphorylated and non-phosphorylated model peptides examined. (**C**) CLA-CL reflecting the H_2O_2-dependent $O_2^{\bullet-}$-generating reaction catalyzed by $ErkG_5H$ and its phosphorylated derivatives. (**D**) Comparison of $O_2^{\bullet-}$-generating activity among the $ErkG_5H$ and its phosphorylated derivatives (error bars, SE, n = 4). (**E**) Positional effect of phosphorylation against the Cu-Tyr interaction. Circled "P" stands for a phosphate residue (**A, B, E**). Graphs and illustrations originally appeared in[9,15] were modified.

$HO^{\bullet}$ generated in the biological system hardly migrates even a short distance in aqueous phase due to its hyper-reactivity toward neighboring molecules such as water molecules, suggesting that $HO^{\bullet}$ generated at the site vicinal to DNA likely results in enhanced degradation of or damages to DNA. Accordingly, the complex between Cu and aforementioned Z-DNA domains readily results in oxidative damaging to DNA chains.[91]

Aiming at prevention of the Cu-mediated DNA damages, which is highly important from a gerontological point of view, Yokawa et al.[92] have reported their attempt in which copper-binding tripeptides with X-X-H motif found within PrP-derived catalytic peptides, prevented the degradation of DNA proceeding in the presence of Cu (II) + H_2O_2 and Cu(II) + ascorbate. The likely mechanism of peptide-mediated prevention of DNA degradation may involve the chelation of the Fenton catalysts such as Fe and Cu ions.

According to Kageenishi et al.,[77] PrP-derived redox-inert copper-binding octapeptide, K-T-N-M-K-H-M-A, effectively protected the plant cells from the toxicity of copper which initiates the apoptotic cell death development in plant cells.

Protection of plant cells by oligo-DNA interacting with metals

Treatments of living plants with excess of transition metals reportedly coincide the production of, molecular responses to, and cellular damages by ROS members such as $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$, possibly *via* direct e^- transfer involving metal cations, formation of catalytic complex with natural chelating agents such as small peptides, or as a consequence of metal-mediated inhibition of metabolic reactions.[93,97] It has been well documented that homeostasis and signaling crosstalk involving ROS and other signaling events such as the changes in $[Ca^{2+}]_c$ are crucial to plant responses and adaptation to abiotic environmental factors threatening the plants, thus determining the fate of plant cells under stressful conditions.[46–48,92,98–100]

We have previously testified the effect of a copper-binding PrP-derived peptide (**Fig. 4A–3**) on protection of tobacco BY-2 cells from Cu toxicity.[77] By analogy, Iwase et al.[101] have shown that oligo DNAs derived from copper-binding motifs function as effective plant cell-protecting agents preventing the toxicity of copper. Accordingly, addition of GC-rich double-stranded DNA fragments (such as CGCGCG hexamer), prior to treatment with copper ions, effectively blocked both the copper-induced calcium

signaling event and resultant programmed cell death. It is known that CGCGCG DNA hexamer is one of minimal GC-rich Z-DNAs.[102]

In case of plant cell protection by Cu-binding peptides, *ca.* 5 to 10-fold higher concentrations of peptides compared to that of Cu^{2+} were required for significantly blocking both the Ca^{2+} influx and cell death induced by copper, simply due to removal of copper with excess of Cu-binding molecule.[77] In contrast, DNA-based protection of plant cells from Cu toxicity requires much lower concentrations of oligo-DNA (between 1/10 and 1/3 of Cu^{2+} concentration) suggesting that there would be alternative mechanism of plant protection performed by added DNA fragments.

Designing the DNA-based catalytic molecules removing $O_2^{\bullet-}$

In addition to simple Cu^{2+} removal model, the DNA-Cu complex examined was shown to possess the $O_2^{\bullet-}$-scavenging catalytic activity, suggesting that DNA-mediated protection of the cells from Cu toxicity is due to removal of $O_2^{\bullet-}$.[101] For assessing the catalytic activity hidden in the Cu-binding DNA oligomers, 4 different sources of $O_2^{\bullet-}$ was employed, namely, (i) short pulse of $O_2^{\bullet-}$ increase by injection of potassium superoxide (KO_2, dissolved in dry DMSO), (ii) slow release of $O_2^{\bullet-}$ in the presence of copper ions and H_2O_2 (through known path for Cu^{2+}-mediated conversion of H_2O_2 to $O_2^{\bullet-}$ *via* HO_2), (iii) addition of SPC-activated $O_2^{\bullet-}$-rich medium which was passed through the SPC reactor equipped with TiO_2-coated alumina fiber, ultra violet light source, and ultrasonic generator designated as Ex-PCAW (as described in **Fig. 1**), and (iv) xanthine oxidase.

Through analysis of $O_2^{\bullet-}$ removal by DNA-Cu complex, the mode of DNA-Cu complex was experimentally proven to be highly similar to the catalytic mode of SOD-like molecules (unpublished results). Kinetic analysis evaluated the V_{max} for the $O_2^{\bullet-}$-degrading action of the DNA-Cu complex, performed in the presence of various concentrations of KO_2 or SPC-dependently produced $O_2^{\bullet-}$, to be between 1.155 and 1.675 nmol / mg DNA / sec.

It is conclusive that the primary role of GC-rich oligo DNA is the trapping of toxic copper ions. Furthermore, upon binding to Cu, such metallonucleic acid complex may show catalytic activity for removal of $O_2^{\bullet-}$. As the V_{max} values obtained from independent assays fell in similar range, we now understand that GC-rich double-stranded DNA forms some catalytic complex for removal of $O_2^{\bullet-}$. This type of molecules could be a good model for designing novel metal-centered artificial nucleozymes, thus fulfilling the criterion predicted by definitive proposition (8):

$$P(Ns) = \exists \{Ns\} \in \{ICs\} \tag{8}$$

Organic catalyst-like biocatalysts

Propositions (1) to (6), (12) and (13) are merely definitive. Predictions by propositions (7) to (9), of the cases of biocatalysts acting upon binding to catalytically active metals, such as copper-centered metallo-enzymes, peptides and nucleic acids, were examined and proven through discussion up to here, thus confirming the generalized proposition (9).

Contrary, our knowledge on the cases fulfilling the proposition (10) is yet to be covered.

$$P(BCs) = \exists \{BCs\} \in \{OCs\} \tag{10}$$

As this proposition predicts that there could be biocatalysts showing catalytic activity due to the action of guanidine-like or amino acid Pro-like catalytic domains without involvement of the action of metals. In addition, the model presented in a series of classical works by Kunitake and his colleagues on imidazole-containing enzyme-like catalytic polymers could be also considered.[103,104]

Interestingly, Pro-containing octarepeat-peptides from human PrP shows self-catalytic generation of $O_2^{\bullet-}$, showing spiky CLA-CL upon mixture with media lacking metals. Since this phenomenon can be silenced by replacing Pro with other groups (Inokuchi et al., unpublished results), this topic may provide more clues to the development of OC-type BCs in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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