Peroxide-Induced Liberation of Iron from Heme Switches Catalysis during Luminol Reaction and Causes Loss of Light and Heterodyning of Luminescence Kinetics.

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SI 1: The "Suicide Inactivation" (Supporting Data)

Figure S 1.1   Dependence of enzymatically catalysed luminol chemiluminescence (eCL) on hydrogen peroxide concentration and pH with HRP as catalyst in the presence and absence of calcium. Luminol luminescence was recorded at various peroxide concentrations and at different pH values. Luminescence integrated over the first 10 min of the reaction is plotted against [H$_2$O$_2$]. A final concentration of 1 µg/ml of HRP was used corresponding to 0.023 µM Fe (pFe = 7.6). All reactions were performed in 100 mM Tris with [LH] = 250 µM and additional 1 mM of CaCl$_2$ (A, B) or 1 mM of EDTA (C, D). B and D are data from A and C, respectively, plotted on a log-log-scale for more detail. Data are averages of four technical replicates. Error bars represent SD. SD is below symbol size, where no error bar can be seen.
Figure S 1.2 Dependence of luminol luminescence on hydrogen peroxide concentration, pH and diverse iron porphyrin compounds as catalysts (eCL). Luminescence integrated over the first 10 min of the reaction is plotted against [H$_2$O$_2$]. All reactions were performed with [LH] = 250 µM in 100 mM Tris at different pH values as indicated in the insets. A: Hemoglobin (4 µg/ml µM corresponding to [Fe] = 250 nM; pFe = 6.6) as catalyst; B: Cytochrome c (10 µg/ml corresponding to [Fe] = 0.8 µM; pFe = 6.1) as catalyst; C: Hemin (2 µM; pFe = 5.7) as catalyst; B, D, F are the double-log plots of A, C, E respectively. Data are averages of four replicates. Error bars represent SD. SD is below symbol size, where no error bars can be seen.
**Table S1**  Dependence of peroxide concentrations giving 50% inhibition (IC₅₀) on the pH with diverse catalysts. Data from **Fig S 1.1** and **S 1.2** above were used to determine the IC₅₀ by sigmoidal four-parametric logistic curve fitting to the data points on the right hand side of the peak. All values are given in µM of H₂O₂. Red framed data from Hb, Cyt c, and Hemin at pH 9 roughly coincide with Soret peak reduction shown in **Figs 11, S 9.1B, and S 9.2B**, respectively.

| Catalyst   | pH = 7  | pH = 8  | pH = 9  | pH = 10 | pH = 11 |
|------------|---------|---------|---------|---------|---------|
| HRP (+Ca²⁺) | 61431   | 6452    | 2347    | 2519    | 3336    |
| HRP (+EDTA) | 38212   | 3107    | 2001    | 2051    | 1812    |
| Hb         | 89757   | 20308   | **5226**| 2919    | 2646    |
| Cyt c      | 98175   | 52295   | **4943**| 2735    | 2747    |
| Hemin      | 67349   | 11039   | **4133**| 3393    | 3276    |
Figure S 2.1  Luminol chemiluminescence catalyzed by iron ions (i.e. iCL) and its dependence on hydrogen peroxide concentration and pH. Luminescence integrated over the first 10 min of the reaction is plotted against various H₂O₂ concentrations. All reactions were performed with [LH] = 250 µM in 100 mM Tris at different pH values as indicated in the insets. A: Luminol reaction with 25 µM FeSO₄ as catalyst (pFe = 4.6); C: Luminol reaction with iron chelate (25 µM FeEDDHA) as catalyst (pFe = 4.6); E: Luminol reaction with 50 µM Fe(NO₃)₃ as catalyst (pFe = 4.3); B, D, F are the double-log plots of A, C, E, respectively. Data are averages of four technical replicates. Error bars represent SD. SD is below symbol size, where no error bars can be seen.
SI 2.2: Non-Heme Iron Proteins as Catalysts of the Luminol Reaction

(Supporting Data)

Figure S 2.2  Luminol luminescence catalyzed by non-heme iron proteins and its dependence on hydrogen peroxide concentration and pH. Luminescence integrated over the first 10 min of the reaction is plotted against various H$_2$O$_2$ concentrations. All reactions were performed with [LH] = 250 µM in 100 mM Tris at different pH values as indicated in the insets. A: Luminol reaction with Ferritin (25 µg/ml) as catalyst; C: Luminol reaction with Ferredoxin (35 µg/ml) as catalyst; B, D are the double-log plots of A, and C, respectively. Data are averages of four technical replicates. Error bars represent SD. SD is below symbol size, where no error bars can be seen.
SI 2.3: The Classic Fenton-Haber-Weiss cycle (Supporting Information).

Dissolved free iron ions, not coordinated by a porphyrin system, and also many other transition metals are able to catalyze the luminol-H\textsubscript{2}O\textsubscript{2}-reaction \textsuperscript{1-3}. As shown (Fig 3 of the main paper and Fig 2.1 above), such catalysis works best under extreme conditions (i.e. [H\textsubscript{2}O\textsubscript{2}] >10 mM; pH > 10). The catalytic efficiency, however, is much lower when compared with porphyrin coordinated iron (Fig S 3 below) and the underlying molecular mechanism is different from the peroxidative cycle, as no heme is involved. The luminol reaction catalyzed by non-porphyrin iron at high pH is incompletely understood. However, the following provides clues as to the reaction mechanism:

With iron and H\textsubscript{2}O\textsubscript{2}, the classic Fenton-Haber-Weiss mechanism (Fig S 2.3) involving hydroxyl radicals (OH\textsuperscript{•}) is an obvious candidate to explain the catalytic effect of iron on the luminol luminescence. Free iron ions usually exist as aqua-iron complexes and the pH determines the prevailing molecular species of this transition metal \textsuperscript{4}.

\[
\text{Fe}^{2+} + \text{H}_2\text{O} \rightleftharpoons [(\text{H}_2\text{O})_6 \text{Fe}^{2+}]^{2+} \quad \text{Eq S 2.1}
\]

At alkaline pH these complexes deprotonate to ferrous hydroxo species, which exist as penta-aqua-complexes \textsuperscript{5}:

\[
[(\text{H}_2\text{O})_6 \text{Fe}^{2+}]^{2+} \underset{\text{pK}=9.51}{\overset{\text{H}^+}{\rightleftharpoons}} [(\text{H}_2\text{O})_5 \text{Fe}^{2+} (\text{OH}^-)]^{+} + \text{H}^+ \quad \text{Eq S 2.2}
\]

In presence of peroxide, ferrous iron is converted into ferric iron in a first step and a hydroxyl radical (OH\textsuperscript{•}) is released \textsuperscript{5,6}:

\[
[(\text{H}_2\text{O})_5 \text{Fe}^{2+} (\text{OH}^-)]^{+} + \text{H}_2\text{O}_2 + \text{H}^+ \quad \overset{\text{Fenton Reaction}}{\overset{(\text{classic})}{\rightarrow}} (\text{H}_2\text{O})_5 \text{Fe}^{3+} (\text{OH}^-) \overset{2^{nd}}{\rightarrow} (\text{H}_2\text{O})_5 \text{Fe}^{3+} (\text{OH}^-) \quad \text{Eq S 2.3}
\]

This process involves several short-lived complex intermediates and peroxide-adducts \textsuperscript{5}. In a second step (Fenton path), ferric iron is recycled back to ferrous iron and hydroperoxy radical (HOO\textsuperscript{•}) is produced \textsuperscript{7}:

\[
[(\text{H}_2\text{O})_5 \text{Fe}^{3+} (\text{OH}^-)]^{2+} + \text{H}_2\text{O}_2 \overset{\text{2nd Fenton Reaction}}{\overset{(\text{classic})}{\rightarrow}} [(\text{H}_2\text{O})_5 \text{Fe}^{2+} (\text{OH}^-)]^{+} + \text{H}^+ \quad \text{Eq S 2.4}
\]
Consequently, the iron in presence of $\text{H}_2\text{O}_2$ switches between the ferrous and the ferric state ($\text{Fe}^{\text{II}} \leftrightarrow \text{Fe}^{\text{III}}$), continuously producing hydroxyl radicals ($\text{OH}^*$) and hydroperoxy radicals ($\text{HOO}^*$). The latter deprotonates to superoxide ($\text{pK}_a = 4.88$) which then transfers its unpaired electron to ferric iron ($\text{Fe}^{\text{III}}$) and is thereby converted to molecular oxygen ($\text{O}_2$) in the course of the Haber-Weiss-reaction:

$$\left[\left(\text{H}_2\text{O}\right)_5\text{Fe}^{\text{III}}(\text{OH}^-)\right]^{2+} + \text{O}_2^* \xrightarrow{\text{Haber-Weiss Reaction}} \left[\left(\text{H}_2\text{O}\right)_5\text{Fe}^{\text{II}}(\text{OH}^-)\right]^+ + \text{O}_2$$  \hspace{1cm} \text{Eq S 2.5}

In summary (Fig S 2.3; Eqs S 2.1 to S 2.5), it is evident that $\text{H}_2\text{O}_2$ is disproportionated by the classic Fenton-Haber-Weiss cycle to water ($\text{H}_2\text{O}$) and molecular oxygen ($\text{O}_2$) while hydroxyl radicals ($\text{OH}^*$) are produced.

**Figure S 2.3**  The classic Fenton-Haber-Weiss cycle. During the classic Fenton-Haber-Weiss cycle hydrogen peroxide promotes redox-cycling of iron between the ferrous ($\text{Fe}^{\text{II}}$) and the ferric ($\text{Fe}^{\text{III}}$) states (red cycle). Hydroxyl radicals ($\text{OH}^*$) are formed when $\text{Fe}^{\text{II}}$ is oxidized (Eq S 2.3) by hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroperoxyl radicals ($\text{HOO}^*$) are formed when $\text{Fe}^{\text{III}}$ is reduced by $\text{H}_2\text{O}_2$ (Eq S 2.4). $\text{HOO}^*$ is deprotonated at high pH ($\text{pK}_a = 4.88$) to superoxide ($\text{O}_2^-$) and protons ($\text{H}^+$) combine with hydroxyl ions ($\text{OH}^-$) to give water. $\text{O}_2^-$ in turn transfers its electron to $\text{Fe}^{\text{II}}$ (Eq S 2.5) and thereby undergoes a conversion to molecular oxygen (Haber-Weiss path in pink).
**SI 2.4: OH•-Scavengers Fail to Inhibit the Luminol Reaction (Supporting Data)**

In the context of the complex luminol reaction (details in the supporting information of Plieth 2018), OH• could be assumed to transfer its radical property to luminol. However, experiments involving OH•-scavengers show, that they do not inhibit the reaction (Fig S 2.4).

![Figure S 2.4](image-url)  
**Figure S 2.4** Dependence of luminol luminescence catalyzed by diverse catalysts on OH•-scavengers. Mannitol (A) and DMSO (B) as well known OH• scavengers were tested for their ability to influence the luminol reaction over a wide range of concentrations and with different catalysts. **A:** Mannitol is unable to inhibit the luminol reaction. **B:** DMSO also does not inhibit the luminol reaction. The observed increase of luminescence with free iron as catalyst and with increasing [DMSO] (yellow symbols) is due to a solvent effect and in agreement with Gorsuch and Hercules (1972) and Ikariyama et al. (1985). Assay conditions: 100 mM Tris/HCl, pH = 9, [LH•] = 750 µM, [H2O2] = 1.1 mM and the amount of catalyst indicated in the inset. The luminescence data were normalized by the luminescence without the added scavenger. Data are averages of three technical replicates. Error bars represent SD. SD is below symbol size, where no error bars can be seen.

This negative result is in agreement with the study of Wong and Salin. It can be explained since OH• as well as free Fe3+-ions are unlikely to exist under the alkaline conditions needed for the luminol reaction. Therefore, the classic Fenton-Haber-Weiss cycle depicted above (Fig S 2.3) is unlikely to occur during the luminol reaction at alkaline pH.
SI 2.5: The Modified Fenton-Haber-Weiss Cycle (Supporting information)

It is more likely that iron bound hydroxyl radical adducts \(^5,6\) and oxo-ferryl iron intermediates \(^13\) exist at high pH. It has been assumed that ferrous hexa-aqua iron is converted by \(H_2O_2\) to a ferrous penta-aqua iron-peroxide intermediate (Eq S 2.6; \(^5\)) which then becomes an oxo-ferryl compound (Eq S 2.7; \(^13\)).

\[
\begin{align*}
\text{[(H}_2\text{O)}_6\text{Fe}^{II}]^{2+} + H_2O_2 & \rightarrow [(\text{H}_2\text{O})_5\text{Fe}^{II}(\text{H}_2\text{O}_2)]^{2+} + H_2O \quad \text{Eq S 2.6} \\
\text{[(H}_2\text{O})_5\text{Fe}^{II}(\text{H}_2\text{O}_2)]^{2+} & \rightarrow [(\text{H}_2\text{O})_5\text{Fe}^{IV}(O^2-)]^{2+} + H_2O \quad \text{Eq S 2.7}
\end{align*}
\]

This ferryl compound could have a sufficiently high oxidation power to produce the luminol radicals (Eq S 2.8) needed for luminescence.

\[
\begin{align*}
\text{[(H}_2\text{O})_5\text{Fe}^{IV}(O^2-)]^{2+} + \text{LH} & \rightarrow [(\text{H}_2\text{O})_5\text{Fe}^{III}(OH^-)]^{2+} + \text{L}^- \quad \text{Eq S 2.8}
\end{align*}
\]

The resulting ferric iron hydroxo complex is then recycled back to ferrous hexa-aqua iron as described before with the classic Fenton-Haber-Weiss cycle (Eqs S 2.4, S 2.2). This modified Fenton-Haber-Weiss cycle explaining the catalysis of the luminol reaction by iron at high pH is schematically summarized in Fig 12 of the main paper.
SI 3: The Light Yield Efficiency of diverse Catalysts (Supporting Data)

The relative light yield $R_{LY}$ calculated as a ratio of photon counts $I_{LY} = \Sigma\text{(counts)}$ to the concentration of the catalytic transition metal [TM] is a reasonable measure for the number of photons, each transition metal atom is able to produce during the luminol reaction ($\text{Eq S 3.1}$). Hence, the lower the concentration of the catalyst [TM] needed to produce $I_{LY}$, the higher is $E_{LY}$.

The logarithm of $R_{LY}$ is defined here as relative light yield efficiency $E_{LY}$ of the luminol reaction. It is used to quantify and compare different catalysts ($\text{Eq S 3.2}$):

$$R_{LY} := \frac{I_{LY}}{[TM]}$$  \hspace{1cm} \text{Eq S 3.1}

$$E_{LY} := \log(R_{LY}) = \log(I_{LY}) + pTM$$  \hspace{1cm} \text{Eq S 3.2}

$pTM$ is defined (like pH) as the negative decadic logarithm of the molar transition metal catalyst concentration [TM]. In particular, when the relevant transition metal is iron, then:

$$E_{LY} = \log(I_{LY}) + pFe$$  \hspace{1cm} \text{Eq S 3.3}

**Figure S 3**  pH-dependencies of light yield efficiencies. Efficiencies calculated as specified above were obtained with different pH values. Assay conditions: 750 µM Luminol; 100 mM Tris/HCl pH = 9; $[\text{H}_2\text{O}_2] = 1.1$ mM. For FeEDDHA, Fe(NO$_3$)$_3$, and Hemin, the total iron concentration equals the molarity of the respective substance. For proteins the Fe-molarities were calculated as follows according to the molecular weight of the protein and the number of Fe bound to each protein molecule: 1 µg/ml of Hb = 0.0625 µM of Fe; 1 µg/ml of Cyt c = 0.081 µM of Fe; 1 µg/ml of HRP = 0.023 µM of Fe. Each bar represents an average of at least three independent experiments and is the mean of at least 15 individual reactions. Error bars represent SD.
SI 4: The Effect of Iron Chelators and Divalent Cations on the "Suicide Inactivation"

(Supporting Data)

Figure S 4  Dependence of luminol chemiluminescence on hydrogen peroxide concentration and the presence of cations and EDTA. Luminescence integrated over the first 15 min of the reaction is plotted against H$_2$O$_2$ concentration. Assay conditions: Tris/HCl 100 mM; pH = 9; 750 µM Luminol; and extra Ca$^{2+}$, Mg$^{2+}$, or EDTA as indicated in the inset. A: Luminol reaction with 30 µg/ml Cyt c as catalyst; B Luminol reaction with 5 µM hemin as catalyst. The reaction was started by injecting a mix of catalyst and luminol into the buffer volume containing H$_2$O$_2$ so that the indicated final [H$_2$O$_2$] was reached.
SI 5: Definition and Modeling: "Glow-Type"- versus "Flash-Type"-Luminescence

(Supporting Information)

In a first approach, chemiluminescence can be considered a redox reaction between a reducing and an oxidizing substrate (Eq S 5.1). The reducing luminogenic substrate $S_{\text{lum}}$ is oxidized by the oxidizing (co-)substrate $S_{\text{co}}$. An excited product $S_{\text{lum}}^*$ is formed which causes the emission of light ($h \cdot \nu$).

$$S_{\text{co}}^{\text{ox}} + S_{\text{lum}}^{\text{red}} \xrightarrow{\text{catalyst}} S_{\text{co}}^{\text{red}} + S_{\text{lum}}^{\text{ox}}^{*} \rightarrow S_{\text{co}}^{\text{red}} + S_{\text{lum}}^{\text{ox}} + h \cdot \nu \quad \text{Eq S 5.1}$$

Usually, the reaction equilibrates and light emission saturates at a certain light level ($L_g$) when the reaction is catalyzed. This requires the reaction to have sufficient substrate or to be continuously supplied by an auxiliary reaction. A reaction running at equilibrium only starts to decay when one of the two substrates ($S_{\text{ox}}$ or $S_{\text{lum}}$) runs out.

"Glow-Type" Luminescence

Long lived light emission is called glow-type luminescence. It can be described by a single exponential function (Eq S 5.2; Figs S 5.1 and S 5.2). The arbitrary time-point when the reaction was started by mixing substrates and catalyst is $t_0$, and the time constant $\tau_g$ determines how fast the luminescence intensity $L(t)$ approaches its equilibrium level $L_g$ after $t_0$.

$$L(t) = L_g \cdot \left(1 - e^{-\frac{(t-t_0)}{\tau_g}}\right) \quad \text{Eq S 5.2}$$

Figure S 5.1    Glow-type luminescence is a simple inverse exponential decay saturating at a certain amplitude $L_g$ as given by Eq S 5.2. The time constant $\tau_g$ is the time needed for the signal to reach 63.2% of $L_g$. 

\[ A_g = 1 \]
\[ t_0 = 10 \]
\[ \tau_g = 10 \]
"Flash-Type" Luminescence

Flash-type kinetics, in contrast, can occur when one of the substrates quickly runs out, when the catalyst is consumed, or when, for some reason, the molecular environment (e.g. pH; [O₂], ROS) changes to the disadvantage of the reaction. Flash-type kinetics can be described by a simple exponential decay (Eq S 5.3). Flash-type kinetics quickly reach a maximum amplitude \( L_f \) when the reaction is started, and then decay exponentially (Figs S 5.3 and S 5.4)

\[
L(t) = L_f \cdot e^{\left( \frac{t-t_0}{\tau_f} \right) - 1}
\]

**Eq S 5.3**

![Exponential decay as given by Eq S 5.3 starting from a certain amplitude \( L_f \). The time constant \( \tau_f \) is the time needed for the signal to come down to 36.8% (= e⁻¹) of \( L_f \).](image)
**Figure S 5.4 Flash-type Luminescence modeled with different time constants.**

**A:** kinetic parameters are: $t_0 = 10$; $L_f = 1,000,000$ and time constants $\tau_i$ are given in the inset. **B:** the same data as in A, however normalized by the area.

**Heterodyning of Flash- and Glow-Kinetics.**

Often, the luminol reaction catalyzed by iron-porphyrin compounds exhibit mixed-type luminescence kinetics. The reaction starts with a flash and then equilibrates at a low glow level. In other words, flash-type fades to glow-type kinetics. The explanation of this is a transition from iron-porphyrin catalysis based on the peroxidase cycle\(^8\) (i.e. eCL) to catalysis by inorganic iron (i.e. iCL) during the reaction. When the heme catalyst is mixed with peroxide of high concentration, then iron is liberated from its porphyrin coordination and converted to a penta-aqua oxo-ferryl catalyst (Eqs S 2.7, S 2.8 above, and Fig 12 of the main paper). This process can mathematically be described by mixing the flash-function (Eq S 5.3) with the glow-function (Eq S 5.2), which leads to biphasic kinetics (Eq S 5.4; Fig S 5.5) as observed in the experiments (Fig 9 of the main paper and Fig S 6 below).

\[
L(t) = L_{\text{max}} \cdot e^{-\frac{(t-t_0)}{\tau_f}} \cdot \left(1 - e^{-\frac{(t-t_0)}{\tau_g}}\right) \tag{Eq S 5.4}
\]

This simplest case of a biphasic kinetic model is determined by just three parameters ($L_{\text{max}}$, $\tau_g$, $\tau_f$). $L_{\text{max}}$ is the maximum amplitude that could be reached and the two time constants, $\tau_g$ and $\tau_f$, determine increase and decay of the signal, respectively.

The time constant $\tau_f$ comprises all depletion processes in the reaction, the depletion of substrates (LH\(^+\) and/or H\(_2\)O\(_2\)), as well as the depletion of heme-catalyst due to the eCL-to-iCL-transition. Thus,
the extent of eCL-to-iCL-transition defines $\tau_r$. A more in-depth modeling would require the splitting of this time constant ($\tau_r$) into three exponentials, each representing a separate depletion process for LH\textsuperscript{-}, H\textsubscript{2}O\textsubscript{2}, and heme-catalyst.

Figure S 5.5 Heterodyne kinetics of luminescence modeled with different decay time constants. A: kinetic parameters are: $t_0 = 10$; $\tau_g = 10$; $L_{\text{max}} = 1,000,000$ and decay time constants $\tau_f$ are given in the insets. B: the same data as in A, however normalized by their area.

Generally, glow-type-luminescence can be distinguished from flash-type-luminescence by the difference between $\tau_g$ and $\tau_r$. If $\tau_r$ is orders magnitudes longer than $\tau_g$ then the glow-type character of the kinetic predominates (Fig S 5.5). A main feature is that these functions (Eq S 5.4) always come down to zero (Eq S 5.5), provided the recording time is long enough.

$$\lim_{t \rightarrow \infty} L_{\text{max}} \cdot e^{\left(\frac{t-t_0}{\tau_g}\right)} \cdot \left(1 - e^{\left(\frac{t-t_0}{\tau_r}\right)}\right) = 0 \quad \text{Eq S 5.5}$$

This implies that the distinction between glow-type and flash-type luminescence is also influenced by the duration of light recording. If the recording period is short, then a long lasting decay cannot be registered and the dataset obtained is specified as glow-type. As a rule of thumb, luminescence can be called flash-type when light decays during the recording period to less 63.2% (= 1 – e\textsuperscript{-1}) of the intensity maximum reached during the experimental recording period.

Biphasic flash-glow luminescence kinetics degenerate to monophasic flash kinetics after addition of EDTA to the luminol reaction as demonstrated by the experiments shown in the main paper (Fig 10) and below (Fig S 7).
SI 6: Kinetics of Luminol Chemiluminescence (Supporting Data)

Figure S 6     Flash type kinetics of luminol chemiluminescence catalyzed by heme proteins (i.e. eCL). The reactions were started at t = 134 s by injecting a mixture of luminol and heme protein into assay buffer with H$_2$O$_2$ to give the final assay conditions: [LH] = 250 µM; pH = 9; [H$_2$O$_2$] as indicated in the insets. A:Kinetics in a semi-log plot obtained from Hb (4 µg/ml which corresponds to 0.25 µM of iron or pFe = 6.6) C:Kinetics in a semi-log plot obtained from Cyt c (10 µg/ml which corresponds to 0.81 µM of iron or pFe = 6.1) as catalyst. B and D: For linear representation, the data shown in A and B were normalized by the area under the curve. This allows a direct comparison of the kinetics and shows that there is a flash-type luminescence at all H$_2$O$_2$ concentrations tested. However, all kinetics also have a glow-type component which becomes more pronounced at high [H$_2$O$_2$] and indicates the liberation of iron from heme and thus a transition from peroxidative catalysis to a modified Fenton-Haber-Weiss catalysis (Eq S 2.6 to Eq S 2.8 above and Fig 12 of the main paper). The diagram insets in B and D give magnifications of the ordinate.
SI 7: The Effect of Iron Chelators and Divalent Cations on Luminescence Kinetics

(Supporting Data)

Figure S 7 Kinetics of luminol luminescence at high [H$_2$O$_2$] and in presence and absence of divalent cations. At high [H$_2$O$_2$] free iron is quickly liberated from heme. The liberated iron catalyzes the chemiluminescence reaction via a modified Fenton-Haber-Weiss mechanism (Eq S 2.6 to Eq S 2.8 above and Fig 12 of the main paper) and produces glow-type kinetics which superposes the flash-type kinetic produced by the heme-catalyst. In presence of EDTA (red curve) the glow-type component in the kinetics is abolished, because liberated iron is chelated, and only the flash-component remains. Assay conditions: Tris/HCl 100 mM; pH = 9; 750 µM Luminol; extra Ca$^{2+}$, Mg$^{2+}$, or EDTA as indicated in the inset. [Cyt c] = 30 µg/ml (A) and [Hemin] = 5 µM (B) were applied as catalyst. The reactions were started by injection of H$_2$O$_2$ to a final concentration of [H$_2$O$_2$] = 100 mM. Kinetics are from the data-set already presented in Fig S4. Data are normalized by the peak value.
SI 8: The Transition of Catalysis as Observed by Kinetics at Different Luminol Concentrations (Supporting Data)

Kinetics obtained under mild conditions ([H$_2$O$_2$] = 0.8 mM) and harsh conditions ([H$_2$O$_2$] = 100 mM) and with different luminol concentrations demonstrate the significance of substrate balance (i.e. ratio of [Luminol] and [H$_2$O$_2$]) and the protective effect of luminol (Fig S 8). At low [H$_2$O$_2$] a glow is produced when [Luminol] is high (Figs S 8A, B). This changes to flash type kinetics when [Luminol] is lower, and indicates exhaustion of luminol. At high [H$_2$O$_2$], in contrast (Figs S 8C, D), an increasing glow is produced when [Luminol] is low (as shown in Fig 8 of the main paper) because iron is liberated from heme and launches a modified Fenton-Haber-Weiss-based catalysis. This effect is reduced with increasing [Luminol] and reveals its protective effect on the catalytic porphyrin core of HRP as reported previously.

Figure S 8  Dependence of luminescence kinetics on [H$_2$O$_2$] and [LH] with HRP as catalyst. A: Kinetics of luminescence obtained with different luminol concentrations under mild conditions ([H$_2$O$_2$] = 0.8 mM) reveal flash type kinetics. C: At strong peroxide concentration ([H$_2$O$_2$] = 100 mM) a first flash indicating peroxidase inactivation is followed by increasing glow-type luminescence revealing the liberation of iron from heme and a transition from enzymatically catalyzed chemiluminescence (eCL) to inorganically catalyzed chemiluminescence (iCL). The higher the luminol concentration the less is this transition effect. B and D: Data shown in A and C, respectively, were normalized by the area under the curve and plotted on linear scale for better comparison of the kinetics. Assay conditions: 100 mM Tris/HCL pH = 9; [HRP] = 2.5 µg/ml (⇒ [Fe] = 0.0575 µM; pFe = 7.24)
SI 9: Soret Peak Monitoring in Presence of Peroxide (Supporting Data)

Figure S 9.1  Absorbance of Cytochrome c in the Soret-Band range in dependence on [H₂O₂].
A: The absorbance was scanned around the typical Soret-Band of Cyt c (i.e. λ = 408 nm) in presence of H₂O₂ at concentrations (µM) as indicated by the inset. B: A(408 nm ±10) plotted against the respective [H₂O₂]. The EC₅₀ was obtained by four-parametric logistic curve fitting. Data in B represent averages of n = 9; error bars indicate SD. Assay conditions: 100 mM Tris/HCl, pH = 9 at 28°C; [Cyt c] = 0.5 mg/ml; [H₂O₂] in (µM) as indicated by the abscissa; 1 h pre-incubation at 28°C before spectral recording.

Figure S 9.2  Absorbance of Hemin in the Soret-Band range in dependence on [H₂O₂]. A: The absorbance was scanned around the typical Soret-Band of Hemin (i.e. λ = 388 nm) in presence of H₂O₂ at concentrations (µM) as indicated by the inset. B: A(388 nm ±10) plotted against the respective [H₂O₂]. The EC₅₀ was obtained by four-parametric logistic curve fitting. Data in B represent averages of n = 9; error bars indicate SD. Assay conditions: 100 mM Tris/HCl pH = 9 at 28°C; [Hemin] = 62 µM; [H₂O₂] in (µM) as indicated by the abscissa; 1 h pre-incubation at 28°C before spectral recording.
SI 10: The Order of Substrate Addition and its Effect on Luminescence Kinetics

(Supporting Data)

Figure S 10  Kinetics of luminol luminescence depend on the order of substrate addition. The luminol reaction was started by successively injecting the two substrates at \( t_1 = 70 \) s and \( t_2 = 170 \) s into the reaction buffer containing the catalyst. Blue traces represent luminol kinetics obtained with luminol injected before \( \text{H}_2\text{O}_2 \) and red traces represent luminol kinetics obtained with inverse injection order (i.e. \( \text{H}_2\text{O}_2 \) injected before luminol). Injection of \( \text{H}_2\text{O}_2 \) before luminol leads to partial destruction of the heme complex, liberation of iron and consequently a reduced light yield and glow kinetics. Flash kinetics are obtained when luminol is injected first and the reaction initiated by addition of \( \text{H}_2\text{O}_2 \). Assay conditions: 100 mM Tris/HCl; pH = 9; \([\text{LH}_2\text{O}_2]\) = 0.6 mM; \([\text{H}_2\text{O}_2]\) = 3.5 mM; (A) \([\text{HRP}] = 1.8 \mu\text{g/ml}]; (B) \([\text{Cyt c}] = 3 \mu\text{g/ml}]; (C) \([\text{Hb}] = 12 \mu\text{g/ml}]; (D) \([\text{Hemin}] = 1.2 \mu\text{M}]; Data represent averages of \( n = 12 \) technical replicates. Error bars represent SD.
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