Hypocrates is a genetically encoded fluorescent biosensor for (pseudo)hypohalous acids and their derivatives

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The lack of tools to monitor the dynamics of (pseudo)hypohalous acids in live cells and tissues hinders a better understanding of inflammatory processes. Here we present a fluorescent genetically encoded biosensor, Hypocrates, for the visualization of (pseudo) hypohalous acids and their derivatives. Hypocrates consists of a circularly permuted yellow fluorescent protein integrated into the structure of the transcription repressor NemR from Escherichia coli. We show that Hypocrates is ratiometric, reversible, and responds to its analytes in the 10^6 M⁻¹s⁻¹ range. Solving the Hypocrates X-ray structure provided insights into its sensing mechanism, allowing determination of the spatial organization in this circularly permuted fluorescent protein-based redox probe. We exemplify its applicability by imaging hypohalous stress in bacteria phagocytosed by primary neutrophils. Finally, we demonstrate that Hypocrates can be utilized in combination with HyPerRed for the simultaneous visualization of (pseudo)hypohalous acids and hydrogen peroxide dynamics in a zebrafish tail fin injury model.

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The past decades have considerably increased our knowledge on the role of reactive oxygen species (ROS), particularly of hydrogen peroxide ($\text{H}_2\text{O}_2$), in physiological and pathophysiological processes. Indeed, $\text{H}_2\text{O}_2$ went from being perceived exclusively as an oxidative stress molecule to a secondary messenger compound that regulates cellular signal transduction pathways by modifying cysteine residues in proteins$^1$–$^3$. Other oxidants of physiological relevance include (pseudo)hypohalous acids, known to participate in immune response reactions. However, specific details regarding their spatio-temporal dynamics and contribution to various aspects of metabolism remain largely unexplored. (Pseudo)hypohalous acids, such as hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypothyiocyanic acid (HOSCN), are produced by a number of mammalian peroxidases: myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO)$^4$. These enzymes catalyze the conversion of (pseudo)halide ions ($X^-$) to $\text{OX}^-$ in the presence of $\text{H}_2\text{O}_2$. The formed HOCl and HOBr can react with nucleophiles containing nitrogen and sulfur atoms, for example, with amines and thiols, as well as with aromatic rings and unsaturated bonds in organic molecules. In a cellular context, this means that possible targets for HOCl and HOBr include different amino acids in proteins, glutathione, lipids, carbohydrates, and nucleobases$^5$–$^8$. As already mentioned, (pseudo)hypohalous acids are one of the key players in immune system functioning. Reactive chlorine species generated by MPO from neutrophils provide defense against bacterial and fungal infections$^9$,$^{10}$. HOBr produced by EPO from eosinophils is a powerful agent used for the destruction of larger parasites$^{11}$, while HOSCN from LPO acts as a potent antibacterial compound primarily in saliva, tears, milk, and Airways$^{12}$,$^{13}$. Levels of (pseudo)hypohalous acids need to be controlled, as their increase is often associated with pathological conditions such as atherosclerosis, diseases of the cardiovascular system and lungs, autoimmune diseases, Alzheimer’s disease, and many others$^{14}$–$^{17}$. However, our knowledge about the precise mechanisms of their regulation, and, more broadly, of how they participate in cellular signaling, is rather limited. It is important to note that HOCl/HOBr and HOSCN differ markedly in their reactivity, with HOSCN being a much weaker oxidant and dismutase$^5$–$^8$. As a result, HOCl/HOBr and HOSCN differ markedly in their mechanisms of their regulation, and, more broadly, of how they participate in cellular signaling, is rather limited. It is important to note that HOCl/HOBr and HOSCN differ markedly in their reactivity, with HOSCN being a much weaker oxidant and dismutase$^5$–$^8$. As a result, HOCl/HOBr and HOSCN differ markedly in their mechanisms.

Hypocrites (NemR-cpYFP biosensor) architecture and design. As a first step, we searched for prokaryotic transcription factors which sense hypochlorite anions ($\text{ClO}^-$). Out of all possible candidates, HypR and NemR were selected$^{42}$,$^{43}$. For our work, a NemR mutant with all Cys residues substituted for Ser, except for Cys106 (NemR$^{\text{ClO}_6}$)$^{42}$, was used to avoid undesirable sensitivity for reactive electrophilic species (RES) and to minimize other non-specific redox reactions, such as disulfide-linked dimerization$^{45}$. To determine which of the two candidates was more suited for our biosensor design, we measured the second-order rate constants of NemR$^{\text{ClO}_6}$ and HypR by monitoring the change of their respective intrinsic tyrosine and tryptophan fluorescence with increasing $\text{NaClO}$ concentrations (Supplementary Fig. 1). We found that NemR$^{\text{ClO}_6}$ (~3.0 × 10$^5$ M$^{-1}$s$^{-1}$) reacts ~200-times faster compared to HypR (~1.5 × 10$^4$ M$^{-1}$s$^{-1}$), while exposure to $\text{H}_2\text{O}_2$, had no effect in both cases (Supplementary Fig. 2). The negative y-intercept might be the result of several chlorination/oxidation events with some of these modifications not directly being within the microenvironment of the tryptophan, which was used as the reporter amino acid for determining the second-order rate constant of NemR$^{\text{ClO}_6}$.

Based on these observations, we selected NemR$^{\text{ClO}_6}$ as a molecular platform to design a biosensor for HOCl detection. NemR$^{\text{ClO}_6}$ consists of a DNA-binding and a sensory domain (Fig. 1a). The latter has a flexible loop with the crucial Cys106 buried in a hydrophobic pocket surrounded by Trp167, Leu168, and Val1109. Notably, its measured reaction rate with $\text{NaClO}$ is ~1000 times slower compared to what has been published for a...
Fig. 1 Hypocrates (NemR-cpYFP biosensor) design and spectral characteristics. a The structure of NemR<sup>C106</sup> (PDB ID: 4YZE) shows the N-terminal DNA-binding domain (colored blue), the C-terminal sensory domain (colored cyan), Cys106 and Lys175 (colored yellow), and the flexible loop (colored red) into which cpYFP was inserted. The N- and C-termini are indicated with N and C, respectively. b The proposed simplified scheme of NemR-cpYFP biosensors functioning in living cells. c The structure of Hypocrates is presented with NemR<sup>C106</sup> colored blue/cyan, cpYFP colored yellow, the linkers between NemR<sup>C106</sup> and cpYFP colored green, and the flexible loop colored red. The upper numbers represent amino acid numbering corresponding to the intact NemR<sup>C106</sup>, while the lower numbers represent numbering corresponding to the biosensor. d Optical properties of purified Hypocrates protein in PBS. e Hypocrates fluorescence excitation spectra in E. coli cells in reduced and NaOCl-oxidized forms. f Purified Hypocrates (0.5 µM) fluorescence excitation spectrum behavior in the presence of NaOCl in saturating concentration. g Purified Hypocrates (0.5 µM) fluorescence excitation spectrum behavior in the presence of N-chlorotaurine (NCT) in saturating concentration. h HypocratesCS fluorescence excitation spectra in E. coli cells in reduced and NaOCl-oxidized forms. i HypocratesKA fluorescence excitation spectra in E. coli cells in reduced and NaOCl-oxidized forms. Source data are provided as a Source Data file.
free cysteine\(^\text{46}\), which indicates that the local environment strongly determines its reactivity. Cys106 has been observed in two conformations in the crystallographic asymmetric unit, with the transition reportedly caused by HOCl\(^\text{45}\) (Supplementary Fig. 3). To visualize these conformational changes, we introduced cpYFP at several positions within the flexible loop. Similar to other cpYFP-based probes\(^\text{34,38,47}\), we expected that, upon proper conformational coupling, structural shifts in the flexible loop induced by the reaction with HOCl would alter the optical properties of the chromophore (Fig. 1b).

This yielded 12 chimeras, in which cpYFP was separated from NemRC\(^\text{106}\) by a variation of short linkers (SAG/G or SAG/GT) (Supplementary Fig. 4a). We hypothesized that shortening of the cpYFP integration region would improve the conformational coupling and, therefore, signal transmission from the sensory to the reporter unit of the sensor. Therefore, we designed four additional variants with one or two amino acid deletions in the flexible loop.

We recombinantly expressed all chimeras in E. coli and tested the changes in their fluorescence excitation spectra by adding NaOCl to the bacterial suspensions as well as purified recombinant proteins (Supplementary Fig. 4b). We selected the variant that had the maximum response amplitude of ~1.6-fold for further studies and named it Hypocrates (Fig. 1c) (Supplementary Fig. 4c). Purified recombinant Hypocrates protein is characterized by two excitation maxima (~425 nm and ~500 nm) and one fluorescence emission (~518 nm) maximum (Fig. 1d). The estimated brightness of the biosensor lies in the ~4400–13,900 range, depending on both the excitation wavelength and the redox state, which is ~7–22% of the Enhanced Yellow Fluorescent Protein (EYFP) brightness (Supplementary Table 1). The addition of NaOCl to E. coli cells expressing Hypocrates results in a ratiometric change in the fluorescence excitation spectrum (Fig. 1e). Thus, the biosensor signal can be expressed as an Ex500/Ex425 ratio. Recombinant purified Hypocrates protein behaves similarly and the ratiometric response can be almost completely reversed by the addition of a reducing agent (Fig. 1f). Treatment of the purified protein with NaOBr, another hypohalite salt, elicited a comparable signal shift of the probe (Supplementary Fig. 5). Next, we decided to test whether Hypocrates is also sensitive to the HOCl-derivative, N-chloroaurine (NCT). Taurine is one of the most abundant amino acids in many tissues\(^\text{48}\). This is especially the case in neutrophils, making NCT one of the most common derivatives of reactive nitrogen species (RNS)\(^\text{49}\).

The selectivity of Hypocrates. We showed that Hypocrates is highly sensitive to (pseudo)hypohalous acids and their derivatives. It is noteworthy that high concentrations of NaOCl and NaOBr (~100 µM at a protein concentration of 0.5 µM), but not NCT, led to pronounced fluorescence quenching due to apparent protein damage, which further indicates that NCT reacts with the sensor in a more specific way (Supplementary Fig. 5). In addition, it is noteworthy that global structural changes of Hypocrates occurred in the presence of NaOCl using circular dichroism (CD). After adding NaOCl to the biosensor, we observed an increase of the molar ellipticity\(^\text{9}\) at 208 nm and 222 nm and a decrease at 194 nm (Fig. 2a). Upon H₂O₂ addition, no CD spectral changes were observed (Supplementary Fig. 6). To test whether the optical shift could be restored, we incubated the NaOCl-oxidized Hypocrates with the reducing agent DTT. After 5 min incubation with DTT, the spectrum of the oxidized biosensor showed a similar pattern as the one of the reduced form (Fig. 2b), indicating the reversibility of the structural changes.

As for other cpYFP-based biosensors (except HyPer\(^\text{238}\)), the ratiometric response of Hypocrates is pH-dependent (Fig. 2c). The pKa of purified Hypocrates is 9.10, and HypocratesCS has a pKa of 9.20. In the presence of NaOCl and NCT, the pKa of the sensor decreases to 8.90 and 8.84, respectively. We measured the ratiometric signal of Hypocrates in buffer solutions with different acidity in the function of increasing NCT concentration. Within the physiological range of pH fluctuations, the shape of the titration curve is stable (Supplementary Fig. 7). However, a shift in pH from 6 to 8 results in a 12-fold signal increase; therefore, appropriate pH controls, such as HypocratesCS, are required for proper data interpretation.

Next, we tested the selectivity of Hypocrates. We incubated the sensor with aliquots of various common oxidants (Fig. 2d). Only minor signal fluctuations were observed in the presence of high concentrations of H₂O₂, xanthine oxidase/xanthine system (O₂\(^\text{−}\) generator), MAHMA NONOate (NO\(^•\) generator) and GSSG (Fig. 2d). However, Hypocrates showed a ratiometric response to ONOO\(^−\) (Fig. 2d) (Supplementary Fig. 5). Therefore, if pronounced reactive nitrogen species production in the system can be expected, implementation of appropriate controls might become necessary. As active electrophiles have been shown to affect the DNA-binding affinity of NemR, as well as the expression of the nemRA–gloA operon\(^\text{45}\), we also tested whether glyoxal, formaldehyde, and methylglyoxal would elicit the response of Hypocrates (Fig. 2d). We found that Hypocrates is insensitive to these compounds.

To validate whether the NaOCl-induced fluorescence changes are NemR\(^\text{106}\)-derived, we treated purified cpYFP (0.5 µM) and two other cpYFP-based biosensors (HyPer-2\(^\text{35}\) and SypHer3s\(^\text{50}\), 0.5 µM) with NaOCl (5–10 µM). HyPer-2 and SypHer3s showed no response. At the same concentration of NaOCl (5 µM), a slight decrease in the intensity of cpYFP fluorescence was detected. This can be explained by bleaching since a single cpYFP has a more open conformation, making the chromophore more accessible to the environment. With a significant increase in the concentration of NaOCl (up to 105 µM), the fluorescence is almost completely quenched. This change in the signal is practically irreversible as...
NemRC106 (2 µM) was determined in the presence of oxidizing λ on the pH value of the buffer solution. NCT, and NaOBr caused Trp-degradation of the NemR-derived domain in Hypocrates gained the ability to sense this compound due to the integration of cYPFP. This change in Trp-fluorescence is not related to direct oxidation of the Trp residue, which we could confirm by incubation with DTT, which indicates that non-specific degradation of the fluorescent protein proceeds under the tested conditions (Supplementary Fig. 8). As such, we concluded that cpYFP itself does not contribute to the ratiometric response. To obtain more direct evidence that the generated signal is due to HOSCN, which might be connected to its higher selectivity towards Cys355. The Ex500/Ex417 ratio stabilizes at response conditions (Supplementary Fig. 8). The oxidants NaOCl, NCT, and NaOBr caused Trp-fluorescence shifts (λem = 295 nm, λem = 350 nm), while HOSCN induced no response. This indicates that the NemR-derived domain in Hypocrates gained the ability to sense this compound due to the integration of cpYFP. This change in Trp-fluorescence is not related to direct oxidation of the Trp residue, which we could confirm with mass spectrometry (Supplementary Table 2).

Hypocrates sensitivity and reaction rates. The sensitivity of Hypocrates towards different oxidants (NaOBr, NaOCl, NCT, and HOSCN) was studied by titrating the biosensor (0.5 µM) with increasing analyte concentrations (up to 15 µM) in sodium phosphate buffer (Fig. 3a–d). Hypocrates reaches saturation at ~4–5 µM (8–10:1 oxidant/sensor ratio) in the cases of NaOBr, NaOCl, and NCT, and at ~1 µM (2:1 oxidant/sensor ratio) in the case of HOSCN, which might be connected to its higher selectivity towards Cys355. The Ex500/Ex417 ratio stabilizes at response values of ~1.8-fold (for NaOCl), ~2.0-fold (for NaOBr), ~1.8-fold (for HOSCN), and ~1.7-fold (for NCT) under saturating conditions. The highest sensitivities were observed for HOSCN and NaOBr, which were ~11.1 µM−1 and ~0.60 µM−1, respectively. This outcome shows that the biosensor is slightly more sensitive to HOSCN and NaOBr than to NaOCl and NCT. To estimate corresponding limits of detection (LOD), we implemented the 3Sb/b approach, where Sb is the residual standard error and b is the slope of the linear regression model. In the described system, the LOD values are ~100 nM for NaOBr, 120 nM for HOSCN, 290 nM for NCT, and 330 nM for NaOCl.

To compare the reaction rates of Hypocrates towards NaOBr, NaOCl, and NCT, the second-order rate constants were measured on a stopped-flow instrument (Fig. 3e–g). We found that the biosensor reacts ~100-fold faster with NaOBr (~4.5 × 10^8 M^−1 s^−1) and NaOCl (~1.4 × 10^8 M^−1 s^−1) compared to NCT (~6.1 × 10^7 M^−1 s^−1). NemRC106 is also less reactive to NCT compared to NaOCl (Supplementary Fig. 9), which can possibly be explained by the fact that NCT is a less aggressive compound. A previous study showed that NCT reacts with glyceraldehyde-3-phosphate dehydrogenase at
**Fig. 3 Hypocrates sensitivity and reaction rates.** Changes in the fluorescence excitation spectra of Hypocrates (0.5 μM) obtained by additions of **a** NaOCl or **b** N-chlorotaurine (NCT) aliquots. **c** Titration curves of Hypocrates (0.5 μM) in sodium phosphate buffer obtained by additions of NaOCl, NaOBr, HOSCN, or NCT aliquots. The data are presented as a mean ± SEM (for n > 2), n ≥ 2. The maximum amplitudes of response are 2.0-, 1.8-, 1.8- and 1.7-fold for NaOBr, HOSCN, NaOCl, and NCT, respectively. In the presence of NaOBr, NaOCl, and NCT, the probe is saturated at ~5 μM, and for HOSCN at ~1 μM.

**d** The same data as in **c**, Hypocrates sensitivity towards NaOCl, NaOBr, NCT, and HOSCN is shown. The data are presented as a mean ± SEM (for n > 2), n ≥ 2.

**e–g** Hypocrates reaction rates. Changes in cpYFP fluorescence at > 515 nm cut-off (λex = 485 nm) were measured as a function of time (insert). The curves were fitted to a double exponential to obtain the observed rate constants (kobs/fast), which were plotted as a function of different NaOCl, NCT, or NaOBr concentrations. The second-order rate constants for NaOCl (1.4 ± 0.056) × 10^6 M⁻¹s⁻¹, NCT (6.1 ± 0.3) × 10^4 M⁻¹s⁻¹, and NaOBr (4.5 ± 0.25) × 10^6 M⁻¹s⁻¹ were determined from the slope of the straight line [kfast = koff/[oxidant] + kon]. The data are presented as a mean ± SD (for n > 2), n ≥ 2. Source data are provided as a Source Data file.
By using the intensiometric cpYFP fluorescence as a read-out, we obtain a negative $k_{\text{off}}$ for NaOCl and NaOBr and relatively high $k_{\text{fast}}$ values, which could be explained by the chlorination/bromination on several amino acids and methionine oxidation observed by mass spectrometry (Supplementary Table 3). Especially Tyr106, located close to Cys355, might be involved in coupling the chlorination and bromination to the chromophore (Supplementary Fig. 10).

**X-ray structure of HypocratesCS.** To gain insights into the biosensor architecture and sensing mechanism, we next crystallized Hypocrates and HypocratesCS. Only HypocratesCS gave diffraction-quality crystals. The orthorhombic crystals (C222$_1$, $a = 90.23$, $b = 95.44$, $c = 106.25$, $\alpha = \beta = \gamma = 90^\circ$) contain one molecule of the biosensor per asymmetric unit and diffract to a resolution of 2.2 Å (Supplementary Table 4). The structure (PDB ID: 6ZUI) was solved by molecular replacement, using *E. coli* NemR$_{106}$ (PDB ID: 4YZE) and the cpYFP-based calcium sensor (PDB ID: 3O77) as search models. HypocratesCS consists of a NemR$_{106}$S-based sensor domain (green) and a cpYFP domain (yellow) that contains the p-hydroxybenzylidene-imidazolidinone chromophore (orange), designated as “CR2” in the PDB (Fig. 4a).

**Fig. 4 The structure of HypocratesCS, a cpYFP-based biosensor (PDB ID: 6ZUI).** a The NemR-sensor domain (green) and the cpYFP domain (yellow) are shown. The chromophore (CR2) in the cpYFP β-barrel is shown in orange stick representation. S355 (to which the reactive C355 was mutated to) and K424 (two orientations of the side chain) are shown in red stick representation. The linkers “SAG” and “GT” are colored blue. The N-terminal residues 1-7 and segments (residues 40-42 and 193-207) are missing. b Superposition of NemR$_{106}$ (blue – PDB ID: 4YZE) with the NemR-sensor domain (green). RMSD - root-mean-square deviation. c N95 connects the sensor domain (green) with cpYFP (yellow). N95 interacts with the backbone of Q96, F97 of the NemR-sensor domain and with S166 of the cpYFP domain. The 4-hydroxybenzyl group of CR2 interacts with the phenyl-ring of F164 over a distance of 3.8 Å. d The imidazolinone ring of CR2 interacts with R303, Q301, V268, E183, and T269.

300 M$^{-1}$s$^{-1}$, and with creatine kinase at $1.2 \times 10^2$ M$^{-1}$s$^{-1}$, making Hypocrates 100 times more efficient in recognizing HOCl-modified taurine.
Hypocrates performance in vitro and in eukaryotic cell culture. We tested the ability of Hypocrates to visualize myeloperoxidase (MPO) activity in vitro. Incubation of the purified protein (0.5 μM) in the presence of the MPO-H2O2 system leads to a ratiometric response with an amplitude shift of 1.79-fold after 10 min of incubation, while H2O2 even at a physiologically irrelevant high concentration (100 μM) induces only a minor oxidation shift of ~1.1-fold (Fig. 5a, b).

To test whether Hypocrates is functional in a eukaryotic system, we expressed the sensor in HeLa Kyoto cells and visualized the signal using fluorescence microscopy. To evaluate the sensitivity of the probe, we tested increasing concentrations of NaOCl and calculated the response as an Ex 500/Ex425 ratio. The minimal NaOCl addition that induced detectable changes of the sensor fluorescence was ~4.2 nmol/(10^5 cells) (Fig. 5c). Exposure to 17 nmol/(10^5 cells) NaOCl did not significantly affect the signal of both probes (Supplementary Fig. 14b), indicating that the behavior of Hypocrates, observed in this system, specifically reflects a NaOCl-induced response.

Hypocrates monitors changes in the levels of hypohalous acids in vivo. To test Hypocrates in vivo, we induced inflammation using tail fin amputation of zebrafish larvae. A similar experiment with the genetically encoded sensor HyPer34 has previously shown that H2O2 concentration significantly increases in the wound margin and reaches its maximal value ~20 min post amputation34. As both H2O2 and hypohalous acids are involved in inflammation, we decided to simultaneously monitor the production of these compounds in this system. To this end, we combined Hypocrates with HyPerRed37, a red sensor for H2O2 (Fig. 6b). Signals of both indicators increased 15 min post amputation (mpa), then HyPerRed fluorescence decreased while the Hypocrates signal changed much slower. These differences in dynamics are expected, as neutrophils participate in the elimination of the H2O2 gradient due to the reaction catalyzed by MPO35. In parallel, we used HypocratesCS as control. Although HypocratesCS signal also increased, statistical analysis revealed that the difference between the responses of Hypocrates and HypocratesCS was significant. Thus, we demonstrated that Hypocrates is suitable for in vivo imaging with HypocratesCS as a control.

Discussion
Our idea of developing a genetically encoded sensor for (pseudo) hypohalous acids detection was born out of the increasing volume of incoming information about proteins that are specifically modified under conditions of hypohalous stress. We present the Hypocrates probe, which is a genetically encoded fluorescent biosensor for visualizing (pseudo)hypohalous acids in live systems. Hypocrates displays a ratiometric reversible change in signal when interacting with (pseudo)hypohalous acids and their derivatives with minimal response-inducing oxidant concentrations located in the 0.1–0.3 μM range (at a biosensor concentration of 0.5 μM). It is known that neutrophils produce high amounts of reactive chlorine species. However, it is difficult to calculate the exact concentrations of HOCl generated by cells since it quickly reacts with surrounding molecules. It has been reported that at the sites of inflammation, the concentration of HOCl generated by MPO from accumulated immune cells reaches levels of 1–2 mM36. Our biosensor demonstrates similar sensitivities to NaOCl, NaOBr, and HOCl, which is why we are positioning Hypocrates as an indicator for visualizing the dynamics of the total pool of (pseudo)hypohalous acids. This is particularly relevant because (pseudo)hypohalous acids are
produced as a mixture in biological systems. Hypocrates also allows monitoring the dynamics of HOCl derivatives. Chloramines are characterized by longer lifetimes due to decreased reaction rates and altered selectivity profiles with more pronounced specificity for sulfur-containing groups. Due to the high concentrations of taurine in neutrophils, N-chlorotaurine (NCT) is one of the most common derivatives of reactive chlorine species. It might seem that having a reaction rate of ~10^6 M^−1 s^−1 for NaOCl, Hypocrates will not be able to visualize hypohalous stress under all conditions, since it could be outcompeted by other sulfur-containing compounds, as some of them are present at millimolar concentrations and operate at 10^7–10^8 M^−1 s^−1. However, being an extremely reactive compound, HOCl hardly induces its biological effects directly. Apparently, HOCl is locally converted into less aggressive halamines, like NCT, and these compounds, in turn, might affect the cellular metabolism. Therefore, the ability of Hypocrates to sense NCT with a relatively high reaction rate makes it an efficient tool for the visualization of hypohalous stress. Recombinantly expressed and purified Hypocrates did not show any response to the major

**Fig. 5 Hypocrates performance in vitro and in eukaryotic cell culture.**

- **a** Fluorescence excitation spectra of purified Hypocrates (0.5 µM) in the presence of individual myeloperoxidase (MPO), H₂O₂ and the MPO-H₂O₂ system. HOCl generated by MPO, leads to the development of a saturating response, while a physiologically irrelevant H₂O₂ concentration induces only minor signal changes.
- **b** Hypocrates (0.5 µM) signal as a function of time in the presence of individual H₂O₂ and the MPO-H₂O₂ system. HOCl, generated by MPO, leads to the development of a saturating response, while a physiologically irrelevant H₂O₂ concentration induces only minor signal changes.
- **c** The titration curve of Hypocrates in HeLa Kyoto cells exposed to different concentrations of NaOCl (values ± SEM, N = 2 experiments, n ≥ 25 cells per experiment).
- **d** Upper part: Images of Hypocrates in transiently transfected HeLa Kyoto cells exposed to 17 nmol NaOCl/(10^5 cells) at different time points. Scale bar = 50 µm. The lookup table indicates changes in the Ex500/Ex425 ratio. Lower part: Hypocrates (blue line) and HypocratesCS (red line) fluorescence changes in E. coli cells phagocytosed by human neutrophils. The starting point on the graph corresponds to the moment at which individual bacteria are phagocytosed by a neutrophil (values ± SEM, N = 3 experiments, 35 bacterial cells in total for each version of the sensor). Source data are provided as a Source Data file.
common intracellular oxidizing agents. However, we observed spectral changes of the sensor in the presence of ONOO\textsuperscript{−}. The saturating concentration of ONOO\textsuperscript{−} that induced the maximum response of Hypocrates in vitro was \textsim 10\,\mu M at a biosensor concentration of 0.5\,\mu M. Thus, in systems in which high ONOO\textsuperscript{−} production is expected, it is recommended to perform a control series of experiments, for example, using various inhibitors of NO\textsuperscript{•} synthases, in order to assess the contribution of ONOO\textsuperscript{−} to the formation of the biosensor response. The acidity of the medium affects the acid-base equilibrium of the chromophores inside the fluorescent proteins; therefore, the relative abundance of their protonated and deprotonated forms largely depends on the pH of the solution. The dependence on H\textsuperscript{+} concentration is especially pronounced for circular permutants of flavoproteins, including cpYFP that we used, since their chromophores are more accessible to the environment. To inactivate the sensor, we substituted the key Cys355 residue with a Ser and obtained the HypocratesCS variant, which is insensitive to (pseudo)hypohalous acids and their derivatives. Since its spectral properties and pH sensitivity are identical to the parameters of the original indicator, HypocratesCS is the most appropriate pH control.

By analogy with the C355S mutation, we expected that a K424A substitution would completely inactivate Hypocrates. However, the HypocratesKA variant was found to act very similarly to the original sensor in terms of NaOCl sensitivity and response amplitude. This observation together with our mass spectrometry data suggests that the Nem\textsuperscript{R\textsuperscript{C106}}-sensor domain of Hypocrates does not function in a similar way to the E. coli transcription repressor NemR in forming a reversible sulfenamide bond\textsuperscript{42,44}, and that even the oxidation of Cys355 itself could be sufficient for signal changes in Hypocrates (Supplementary Fig. 10). We hypothesize that the oxidation of Cys355 (mass spectrometry data showed sulfinic and sulfonic acid formation – Supplementary Table 3), which is located on a flexible loop next to Gly354, couples to Gln99 and this residue, in turn, couples to Asn95, which is in contact with the 4-hydroxybenzyl group of the chromophore (CR2) via a water molecule. Hence, oxidation of Cys355 mediated by (pseudo)hypohalous acids might induce changes in the hydrogen-bonding network surrounding CR2 that lead to a ratiometric response. Next to Cys355 oxidation, our kinetic and mass spectrometric studies point in the direction of a potential alternative chlorination/bromination mechanism. A potential culprit residue in the active site of the sensing domain, located within a hydrogen-bonding distance of Cys355, is Tyr106 (Supplementary Fig. 10). This tyrosine is found to be chlorinated and brominated (Supplementary Table 3). It is tempting to speculate that the formation of chloramine on the flexible Lys424 (observed in two different side-chain orientations) via Cys355 could catalyze Tyr106 carbon 3 and carbon 5 chlorination\textsuperscript{57–59}. To facilitate this process, a deprotonated sulfur of Cys355, located N-terminally of a short helix, and the Ne2 of His63 would help to delocalize electrons in

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**Fig. 6 Hypohalous acids and H\textsubscript{2}O\textsubscript{2} dynamics during zebrafish larval wounding.** a Hypocrates and HyPerRed imaging. Zebrafish embryos were co-injected with Hypocrates or HypocratesCS and HyPerRed mRNAs at the 1-cell stage, and a tail fin amputation assay was performed on 48 h post-fertilization larvae. Images were taken before amputation, and time lapse imaging was performed up to 60 min post-amputation (mpa). The lookup tables indicate changes in the oxidation states of the sensors. Scale bar = 100\,\mu m. b Hypocrates ratio and HyPerRed fluorescence were quantified at the amputation plane and normalized to the mean fluorescence on the uncut tail for each larva. Ratio quantification on larvae tail fin expressing Hypocrates (blue lines) or HypocratesCS (red lines) is shown. Two-way ANOVA test with a Tukey’s multiple comparisons posttest was used to determine if the observed difference was statistically significant. Non-amputated embryos (dashed lines) expressing Hypocrates or HypocratesCS were also imaged as a control (values ± SEM; N = 4 experiments, n ≥ 3 embryos/timepoint; ns no significant, **P < 0.01, ***P < 0.001, versus HypocratesCS cut larvae, P\text-superscript{-}\text-superscript{values} are shown). For control embryos (dotted lines), fluorescence has been normalized for each embryo to the first image of the time lapse (t = 0). HyPerRed fluorescence quantification on larvae tail fin expressing HyPerRed (black lines) is shown (values ± SEM, N = 3 experiments, n ≥ 7 embryos/timepoint; ***P < 0.001, versus uncut larvae (dashed line), P\text-superscript{-}\text-superscript{values} are shown). Source data are provided as a Source Data file.
the direction of the OH of Tyr106 making both carbons on the Tyr phenol ring more susceptible for chlorination. This chlorination takes place in a region of the sensing domain which could couple to the hydrogen-bonding network surrounding the chromophore resulting in a reversible ratiometric response. However, elucidating the precise sensing mechanism of Hypocrates is the subject of a separate study.

The crystal structure of HypocratesCS is a cpFP-based redox biosensor that reveals its CR2 chromophore environment within its overall structure (Fig. 4a,c). Overall structure comparison of the β-barrel of cpYFP in Hypocrates (PDB ID: 6ZU1 [https://doi.org/10.2210/pdb6ZU1/pdb]) with the calcium biosensor, Case16 (PDB ID: 3O77 [https://doi.org/10.2210/pdb3O77/pdb])43, showed that these β-barrels are very similar (rmsd of 0.28 Å for 190 atoms). Further, both have a CR2 chromophore, while GFP is characterized by a CRO chromophore (Supplementary Fig. 12d). As such, the cpYFP in Case16 is actually a cpYFP, and not a cpGFP as mentioned in Leder et al.43. The structure of HypocratesCS is not only important for understanding the functioning of this biosensor, but also for revealing the features of other cpYFP-based probes, which show subtle differences in their CR2 chromophore environment (Supplementary Fig. 12a, c), as well as for the rational structure-guided design of future cpYFP-based biosensors for other analytes.

As a defense mechanism against pathogens, immune cells generate hypohalous acids. We tested the functioning of Hypocrates by visualizing hypohalous stress in the bacterial cytoplasm of E. coli captured and swallowed by neutrophils. To accomplish this, E. coli cells expressing Hypocrates were added to human peripheral blood neutrophils while the signal of the probe was monitored with a fluorescence microscope. When individual bacteria entered the neutrophils, we registered a sharp increase in the ratiometric response. In the same system, the signal of HypocratesCS also increased, but to a lesser extent. These observations correspond to the previously obtained results from the literature, showing that the human neutrophil phagosome adopts an alkaline pH for several tens of minutes after phagocytosis60,61. Thus, the dynamics of the inactivated HypocratesCS version most likely reflect alkalization of the bacterial cytoplasm. With the use of HypocratesCS as a control, it, therefore, becomes possible to assess the contribution of the pH component to the Hypocrates signal.

Finally, Hypocrates is suitable for studying inflammatory reactions in vivo. In this work, we induced inflammation by injecting the caudal fin of Danio rerio larvae. Previously, it was shown with the HyPer biosensor that an H₂O₂ gradient is formed in the wound, which serves to attract neutrophils to the area of inflammation54. In turn, neutrophils subsequently participate in the elimination of this gradient due to the reaction catalyzed by MPO55. Here, we observed the simultaneous real-time dynamics of H₂O₂ and (pseudo)hypohalous acids in vivo in zebrafish tissues during inflammation using the red fluorescent biosensor HyPerRed37 and green-emitting Hypocrates. This demonstrates how Hypocrates can be combined in a multiparameter microscopy mode with other biosensors with suitable spectral properties to disentangle the interactions between various reactive species involved in the inflammatory response, and beyond.

Methods

Ethics statement. The D. rerio experiments were approved by French Ministry of Agriculture (n°CT3-05-12). The human neutrophil experiments were approved by the local ethics committee of Pyrgos Russian National Research Medical University and conducted in accordance with the Declaration of Helsinki. All blood donors were informed of the final use of their biological materials and signed an informed consent document.

Expression and purification of S. aureus HypR. The pET-11b-HypR plasmid43 was transformed in E. coli BL21 (DE3) cells, which were grown in Lysogeny Broth (LB) at 37 °C until the A600 reached 0.8. Isopropyl-1-thiogalactopyranoside (IPTG; 1 mM) was added for the expression induction, followed by 3.5 h of incubation at 37 °C. Harvested cells were pelleted at 4 °C, 5000 rpm for 15 min using the Avanti® J-26xp centrifuge (Beckman Coulter®) with a JA-20 rotor and resuspended in lysis buffer composed of 20 mM HEPES/NaOH pH 7.5, 0.5 M NaCl, 1 mM dithiothreitol (DTT), 0.1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1 μg/ml Leupeptin, 50 μg/ml Dnase I, and 20 mM MgCl₂. The cells were lysed using a Sonic VibraCell sonicator for 10 min, with 30 s sound/30 s pause with 61% amplitude. Cell debris was removed by centrifugation (18,000 rpm, 4 °C, 1 h). The supernatant was in-batch incubated with Ni²⁺-Sepharose 6 Fast Flow beads (Cytiva) equilibrated with the binding buffer (20 mM HEPES/NaOH pH 7.5, 0.5 M NaCl and 10 mM imidazole) for 1 h at 4 °C. The beads were then packed in a column coupled to an AKTA® Pure system (GE Healthcare, Life Sciences) controlled by UNICORN 6.3.0.731 software. HypR was eluted using a linear gradient with elution buffer: 20 mM HEPES pH 7.5, 0.5 M NaCl and 0–500 mM (0–100%) imidazole. Protein purity was assessed on a non-reducing SDS-PAGE gel, and the pure fractions were dialyzed (20 ml sample/2 l dialysis buffer) overnight at 4 °C against 20 mM HEPES pH 7.5 and 230 mM NaCl, and stored at ~80 °C in 20% glycerol.

Expression and purification of E. coli NemRC106. The pET 21b(-)–NemRC106 plasmid35 which contains only cysteine (Cys106), was transformed in E. coli BL21 (DE3) cells. Cells were grown in LB supplemented with 50 μg/ml of kanamycin at 37 °C until the A600 reached 0.8. IPTG (0.5 mM) was used for the expression induction, followed by 3 h of incubation at 37 °C. Harvested cells were then pelleted at 4 °C, 5000 rpm for 15 min using the Avanti® J-26xp centrifuge (Beckman Coulter) with a JA-20 rotor and resuspended in lysis buffer composed of 50 mM Tris/HCl pH 8, 0.2 M NaCl, 1 mM DTT and 0.1 mg/ml AEBSF, 1 μg/ml Leupeptin, 50 μg/ml Dnase I, and 20 mM MgCl₂. Cells were disrupted and centrifuged as mentioned above. The supernatant was in-batch incubated with Ni²⁺-Sepharose 6 Fast Flow beads (Cytiva) equilibrated with 50 mM Tris/HCl pH 8, 0.2 M NaCl, and 1 mM DTT for 1 h at 4 °C. The beads were packed in a column, and the AKTA® Pure system (GE Healthcare, Life Sciences) controlled by UNICORN 6.3.0.731 software was used for purification. NemRC106 was eluted using a linear gradient with elution buffer consisting of 50 mM Tris/HCl pH 8, 0.2 M NaCl, 1 mM DTT and 0 to 700 mM (0–100%) imidazole. Following purification, protein purity was assessed on a non-reducing SDS-PAGE gel. Protein purity was assessed by dialyzing (20 ml sample/2 l dialysis buffer) overnight at 4 °C against the binding buffer and stored at ~20 °C.

Molecular cloning procedures. Tersus Plus PCR Kit (Evrogen) was used for all amplification procedures. Primers are listed in Supplementary Table 5. An overlap extension PCR protocol was implemented to engineer different NemR-cpYFP versions. Each reaction mix included the NemRC106 N- and C-terminal fragments and cpYFP fragment in equal molar amounts. The DNA construct was estimated with horizontal DNA electrophoresis in an agarose gel. The pQE30-Hyper-2 plasmid35 was used as a template to amplify the cpYFP part. Two versions of this fragment (with SAGG and SAG/GT linker pairs) were generated with the use of the pQE30-Hyper-2 plasmid and was used on a non-reducing SDS-PAGE gel. Protein purity was assessed by dialyzing (20 ml sample/2 l dialysis buffer) overnight at 4 °C against the binding buffer and stored at ~20 °C.
molar amounts. The DNA concentration was estimated with horizontal DNA electrophoresis in an agarose gel. The pQ3E0-Hypocrates plasmid was used as a template to amplify both parts. The N- and C-terminal colonies were generated with the use of N33/N34 and N16/N21 primer pairs, respectively, for HypocratesCS and N33/N35 and N17/N21 primer pairs, respectively, for HypocratesKA. The reaction mix after overlap extension PCR was subjected to the same procedures as described above.

To transfer NemR-cpYFP versions from the pQ3E0 vector to the pCS2+ vector, the corresponding gene was amplified with the use of the N18/N36 primer pair and purified with Cleanup Standard Kit (Evrogen). The obtained construct and intact pCS2+ vector were incubated with Clal and XbaI FastDigest® enzymes in the corresponding buffer (Thermo Scientific) at 37 °C for 20 min. The restricted polynucleotides were then subjected to the same procedures as described above.

**Functionality tests of NemR-cpYFP variants in E. coli cells.** To obtain bacterial cells that express NemR-cpYFP variants, the pQ3E0 vector bearing the desired gene was transformed to E. coli XL1-Blue cells, after which they were grown on LB-agar plates containing 100 µg/ml ampicillin for 14 h at 37 °C. In all cases, the bacterial density was controlled to achieve conditions in which the individual colonies were located at a distance of 2 mm from each other, as this parameter significantly affects the maturation and the redox state of the sensors (~5–10 ng of DNA per plate). The fluorescence intensity of the cells was estimated with the use of an Olympus US SZX12 fluorescent binocular microscope. On the first day, all NemR-cpYFP versions were characterized by weak fluorescence, which was attributed to the fact that the recombinant fluorescent proteins are relatively new constructs and require more time for efficient maturation. Taking into account, the LB-agar plates were incubated for an additional 24 h at 17–20 °C, as it is known that the maturation of circularly permuted fluorescent proteins proceeds better at lower temperatures. To test the functionality of NemR-cpYFP variants, the bacterial biomass was transferred to 1 ml of phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4, pH 7.4, here and after) and resuspended with an automatic pipette. The fluorescence emission spectra (λex = 425 nm or 500 nm) and excitation spectra (λem = 525 nm) were recorded with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer controlled by Cary Eclipse Scan 1.1(32) Application. The suspensions were treated with NaOCl aliquots to achieve a final oxidant concentration of 80 µM, after which the spectral measurements were repeated. In all cases, the samples were mixed by pipetting prior to the final spectra registration until the signal stabilization was observed. The data were analyzed with OriginPro 9.0 (OriginLab).

**Expression and purification of NemR-cpYFP variants, EYFP, intact cpYFP, HyPer-2, and SypHer3s.** In the current work, two different protocols for Hypocrates expression and purification were used. Both of them led to obtaining the functional biosensor. Therefore, they should be considered equal.

Protocol 1. XL1-Blue cells were transformed with pQ3E0-Hypocrates plasmid, after which they were plated (LB-agar medium, 100 µg/ml ampicillin) and incubated for 24 h at 17–20 °C. The bacterial density was controlled as described above. To achieve better protein folding and maturation, the plates were additionally incubated for 24 h at 17–20 °C. Next, the cells were washed from the agar surface by ice-cold PBS, and the final volume of the suspension was adjusted to 24 ml with the same buffer. The number of plates used for a single purification procedure was twenty. The cells were disrupted with the use of a Sonic VibraCell instrument in an ice bath (5 s sonication post 10 s pause cycle; total sonication time ~ 9 min; the amplitude ~ 32%). The obtained lysates were centrifuged for 20 min at 21,000xg and 4 °C (Centrifuge 5424 R, Eppendorf) to precipitate insoluble fractions. The supernatants were collected and applied to a column filled with 5 ml of TALON Metal Affinity Resin (Takara) previously equilibrated with ice-cold PBS. The column was washed with 50 ml of the same buffer to get rid of non-target proteins. The elution step was performed by the addition of 10 ml of ice-cold PBS containing 250 mM imidazole, and the fraction with the target protein was collected on the basis of its bright yellow color. The elimination of imidazole was achieved by gel filtration with a 10 ml of Sephadex G-25 (GE Healthcare Life Sciences) previously equilibrated with ice-cold PBS. The pure protein sample was stored at 4 °C for no more than 3 days. The addition of any reducing agents (such as β-mercaptoethanol) did not alter the properties of the protein – the sensor was obtained in its fully reduced form, even in their absence. Hypocrates samples, purified according to this protocol, were used for the following tests: the measurements of optical parameters, fluorescence spectra stability and reversibility experiments, titration by N-chlorotaurine at different pH values, gel filtration, and measurements of MPO activity. Other primary NemR-cpYFP versions, EYFP, cpYFP, SypHer3s, and HyPer-2 were purified following the same protocol. However, in the case of the latter, all buffers, except for those used at the gel filtration step, contained 5 mM β-mercaptoethanol to avoid the oxidation of the sensor. The protein concentration in the final samples was measured with the use of Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich) and a 96-well plate analyzer (Tecan Infinite 200 PRO) controlled by Tecan i-control 1.1.1.1.1 software.

Protocol 2. Shuffle™ T7 or XL1-Blue cells were transformed with pQ3E0-Hypocrates plasmid, respectively. The cells were plated on LB-agar-ampicillin and incubated overnight at 37 °C (for XL1-Blue) and 30 °C (for Shuffle™ T7). Plates were transferred to a 25 °C incubator until they expressed the protein, as indicated by turbidity. The recombinant colonies were transferred to 3 l of LB medium supplemented with 100 µg/ml ampicillin and incubated for 36 h at 25 °C with shaking at 180 rpm. Cells were harvested, and the pellet was resuspended in lysis buffer composed of 40 mM Tris pH 7.5, 150 mM KCl, 10 mM MgSO4, 5 mM DTT, 0.1 mg/ml ABEF, 5 µg/ml Leupeptin, 50 µg/ml DTT, and 20 mM MgCl2. Cells were lysed and centrifuged as performed for NemR-cpYFP, and the supernatant was in-batch incubated with Ni2+-Sepharose beads (Thermo Scientific) equilibrated with binding buffer: 40 mM Tris pH 7.5, 150 mM KCl, 10 mM MgSO4 and 1 mM DTT for 1 h at 4 °C. After column packing, the AKTA® Purification System (GE Healthcare) was controlled by HypocratesCS software. EYFP was used to elute the protein using a binding buffer with 400 mM imidazole followed by size exclusion chromatography on a Superdex 75 16/60 (GE Healthcare) column equilibrated in binding buffer. The purity of the protein was assessed on a non-reducing SDS-PAGE gel, and the pure fractions were collected and stored at ~20 °C. Hypocrates and HypocratesCS samples, purified according to this protocol, were used for the following tests: circular dichroism experiments, pkA determination, fluorescence selectivity experiments, fluorescence sensitivity experiments, pre-steady-state kinetic measurements, HypocratesCS crystallization, and mass spectrometry.

**N-chlorotaurine, NaOH, HOSCN, and NaONOO preparation.** The preparation of N-chlorotaurine (NCT) was carried out according to Patent DE4041703A1 (https://www.google.com/patents/DE4041703A1). 3-Chloro-2-mercaptoethanol (6.0 g, 21.3 mmol) was dissolved in dry methanol (50 ml). Finely powdered taurine (2.5 g, 20 mmol) was added, and the mixture was stirred for 20 h at room temperature (~20–25 °C). The solvent was removed on a rotary evaporator, and the residue was washed with isopropl alcohol (3 times, 10 ml) and diethyl ether (three times, 10 ml) to remove potential impurities. The white solid obtained in the vacuum (5 mbar) analysis of the product (DMSO-d6) showed the absence of aromatic protons. The product was stored at ~20 °C.

The preparation of NaOH solution was carried out according to Liu et al.32 NaOH (11.7 g, 0.30 mol) was dissolved in water (100 ml). The solution was cooled to ~5 °C in ice bath. The mixture was diluted with 1 ml of 500 mM sodium phosphate buffer pH 7.4 (1:5) with a Space A-100 column equilibrated in binding buffer (10 mM MgSO4 and 1 mM DTT for 1 h at 4 °C). After column packing, the AKTA® Purification System (GE Healthcare) was controlled by HypocratesCS software. EYFP was used to elute the protein using a binding buffer with 400 mM imidazole followed by size exclusion chromatography on a Superdex 75 16/60 (GE Healthcare) column equilibrated in binding buffer. The purity of the protein was assessed on a non-reducing SDS-PAGE gel, and the pure fractions were collected and stored at ~20 °C. Hypocrates and HypocratesCS samples, purified according to this protocol, were used for the following tests: circular dichroism experiments, pkA determination, fluorescence selectivity experiments, fluorescence sensitivity experiments, pre-steady-state kinetic measurements, HypocratesCS crystallization, and mass spectrometry.
respectively) of the samples were recorded with the use of a Varian Cary 5000 Spectrophotometer (controlled by Varian UV Scan Application 3.00(339)) or a Varian Cary Eclipse Fluorescence Spectrophotometer (controlled by Cary Eclipse Scan 1.132 Application). The molar extinction coefficients (ε) were calculated according to the following equation
\[ \epsilon = \frac{A}{C \cdot l} \]
where A was the optical density at the studied absorption maximum, C was the protein concentration (M), and l was the optical path length (cm). The fluorescence quantum yields (QY) were calculated according to the following equation
\[ QY = \frac{I_{\text{em}}}{I_{\text{ex}} \cdot \epsilon} \]
where I_{\text{em}} is the emission intensity at the studied excitation maximum, I_{\text{ex}} is the excitation intensity at the studied absorption maximum, and Em was the emission intensity at the studied excitation maximum (λex = 425 nm or 500 nm for NemR-cpYFP variants, and 519 nm for EYFP), respectively. The fluorescence quantum yields of the samples were recorded with the use of a Varian Cary 5000 Spectrophotometer (controlled by Cary Eclipse Scan 1.132 Application). The data were analyzed with OriginPro 9.0 (OriginLab).

Fluorescence spectra stability and reversibility experiments. To investigate whether high oxidant concentrations damage the proteins, purified Hypocrates samples (0.5 µM) were treated with saturating oxidant concentrations (5–10 µM), and the fluorescence spectra (λex = 295 nm) were recorded. The aliquots of corresponding oxidants were added to achieve extremely high concentrations (105–110 µM), and the same measurements were carried out in all cases. The samples were mixed by pipetting prior to the final spectra registration until the signal stabilization was observed. NaOCl and N-chloroaniline were tested in PBS, while NaNOO and NaNO2 were tested in 100 mM sodium phosphate buffer (pH 7.4) to avoid possible OCl− generation in the system. In the latter two cases, the protein aliquots were buffer-exchanged using Amicon Ultra-0.5 Centrifugal Filter Units (Millipore) or by gel filtration on columns filled with 10 ml of Sephadex G-25 (GE Healthcare Life Sciences) previously equilibrated with ice-cold medium. The NaOCl sensitivity of intact cPYP, SypHer3 and HyPer 2 purified proteins were investigated according to the same protocol. The measurements were performed with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer controlled by Cary Eclipse Scan 1.132 Application. The data were analyzed with OriginPro 9.0 (OriginLab).

To investigate whether Hypocrates oxidation is reversible, purified protein samples (0.5–2 µM) were treated with saturating oxidant concentrations (5–50 µM) and incubated for 5 min, after which the fluorescence excitation spectra were recorded. Next, DTT was added to the reaction mix to the final concentration of 1–3 mM, and the probes were incubated for 40–60 min prior to the spectra registration. In some cases, two additional control probes (intact and with the same oxidant concentration) were prepared and incubated for the same time to control for possible artifacts caused by prolonged atmosphere exposure. NaOCl and N-chloroaniline were tested in PBS, while NaNOO and HOSCN were tested in 100 mM sodium phosphate buffer (pH 7.4) to avoid possible OCl− generation in the system. In the latter cases, the protein aliquots were buffer-exchanged using Amicon Ultra-0.5 Centrifugal Filter Units (Millipore) or by gel filtration on columns filled with 10 ml of Sephadex G-25 (GE Healthcare Life Sciences) previously equilibrated with ice-cold medium. The NaOCl sensitivity of intact cPYP, SypHer3 and HyPer 2 purified proteins were investigated according to the same protocol. The measurements were performed with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer (controlled by Cary Eclipse Scan 1.132 Application) or an LS55 luminescence spectrophotometer (controlled by FL WinLAB 4.00.03). The data were analyzed with OriginPro 9.0 (OriginLab).

Biosensor secondary structural changes with circular dichroism. Changes in the overall secondary structure of Hypocrates between its reduced and oxidized (NaOCl or H2O2) forms were evaluated with circular dichroism (CD) spectroscopy. The protein was treated with 30 mM DTT for 5 min at room temperature, and the CD spectra (with a Hit-Trap® desalting column (GE Healthcare), equilibrated with 20 mM sodium phosphate buffer pH 7.4, was used to remove excess DTT. To prepare the oxidized samples, Hypocrates (250 µM) was incubated for 10 min at room temperature with different concentrations of NaOCl (1, 1.5, and 2.0 mM) or H2O2 (1, 1.5, and 1.6 mM). The spectra were treated with the use of the CD program from 4000 to 200 nm. The CD spectra from each experiment were analyzed using GraphPad Prism® 8 and OriginPro 9.0 (OriginLab).

To determine whether the overall secondary structure could be restored, DTT was used. NaOCl-oxidized Hypocrates (1:10 protein/oxidant ratio) was incubated with 200 µM DTT for 5 min at room temperature. The background from the buffer and the presence of 200 µM DTT were subtracted.

pKa determination of reduced and oxidized NemR-cpYFP versions. To determine the pKa of Hypocrates, the protein was reduced with DTT and buffer-exchanged into 100 mM sodium phosphate buffer (pH 7.4) using a Hi-Trap® desalting column (GE Healthcare). Reduced Hypocrates (0.5 µM) in the presence or absence of 12.5 µM oxidants (NaOCl and NCT) was diluted in a polybuffer solution with several pH values (0.5 pH unit interval) and incubated for 10 min. The excitation spectra (with λem = 555 nm) were recorded after 5 min of incubation at 25 °C using a SpectraMax iD5 plate reader (Molecular Devices) controlled by Softmax Pro 7.1. The polybuffer solution consisted of sodium acetate (10 mM), sodium phosphate (10 mM), sodium borate (10 mM), and sodium citrate (10 mM). The ExcSOFT/ExA170 ratio was plotted as a function of increasing buffer pH. For each measurement, at least three independent replicates were performed, and the data were analyzed using GraphPad Prism® and OriginPro 9.0 (OriginLab). The pKa of reduced HypocratesCS was determined as described for reduced Hypocrates.

Fluorescence selectivity experiments. Prior to the experiment, the purified protein was reduced with DTT and buffer-exchanged using a Hi-Trap® desalting column (GE Healthcare) into 100 mM sodium phosphate buffer (pH 7.4). Aliquots of the sensor (2 µM) were incubated with different oxidants (NaOCl, NaOBr, HOSCN, and NaO2Br) for 5 min at 25 °C. The excitation spectra (λex = 295 nm) were recorded. Next, the fluorescence were measured using a Fluorescence Spectrophotometer (controlled by Cary Eclipse Scan 1.132 Application) or an LS55 luminescence spectrophotometer (controlled by FL WinLAB 4.00.03). The excitation spectra were analyzed using GraphPad Prism® 8 and OriginPro 9.0 (OriginLab).

Fluorescence sensitivity experiments. Hypocrates sensitivity experiments were performed in 100 mM sodium phosphate buffer (pH 7.4). Aliquots of the purified protein (0.5 µM) were incubated with increasing concentrations of oxidants (NaOCl, NaOBr, HOSCN, and N-chloroaniline) for 5 min at 25 °C. The fluorescence were measured using a Fluorescence Spectrophotometer (controlled by Cary Eclipse Scan 1.132 Application) or an LS55 luminescence spectrophotometer (controlled by FL WinLAB 4.00.03). The excitation and emission wavelengths for each protein were λexc = 295 nm, was plotted as a function of increasing oxidant concentration. For each measurement, at least two independent replicates were performed, and the data were analyzed using GraphPad Prism® 8 and OriginPro 9.0 (OriginLab).

Hypocrates were analyzed in 100 mM sodium phosphate buffers (pH 7.0, 7.3, 7.6, and 7.9). Aliquots of the purified protein (0.5 µM) were treated with serial N-chloroaniline additions of 0.3–5 mM up to the final concentration of 15 mM. The fluorescence excitation spectra (λem = 525 nm) were recorded with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer controlled by Cary Eclipse Scan 1.132 Application. At each titration step, the samples were mixed by pipetting prior to the final spectra registration using a SpectraMax iD5 plate reader (Molecular Devices) controlled by Softmax Pro 7.1. The ExcSOFT/ExA170 ratio, which represents the ratio between fluorescence excited at 500 nm and at 417 nm, was plotted as a function of increasing oxidant concentration. The initial linear part of the hyperbolic curve was analyzed using linear regression, where the slope values represent the sensitivity towards the corresponding oxidants. For each measurement, at least two independent replicates were performed, and the data were analyzed using GraphPad Prism® 8 and OriginPro 9.0 (OriginLab).

Pre-steady-state kinetic measurements. Pre-steady-state kinetic measurements were performed using a stopped-flow apparatus coupled to a fluorescence detector (Applied Photophysics SX 20) and controlled by Prodata SX 2.5.0. For HyPer, changes in intrinsic Tyr fluorescence were measured using a > 305 nm cut-off filter (λem = 274 nm). For NemRCS, changes in intrinsic Trp fluorescence were measured using a > 320 nm cut-off filter (λem = 295 nm). For Hypocrates, changes in the cpYFP chromophore fluorescence were measured using a > 515 nm cut-off filter (λem = 485 nm). Excitation and emission wavelengths for each protein were determined prior to the stop-flow experiments using an LS55 luminescence spectrophotometer (PerkinElmer) controlled by FL WinLAB 4.00.03. The
oxidoant concentration range required to lead to fluorescence changes for each protein was also determined. The purity of Hypocrates protein was identified by SDS–PAGE in the presence of samples were reduced with 30 mM DT 3 DTT for 30 min at room temperature. A Hi-Trap desalting column (GE Healthcare), equilibrated with argon-flushed 100 mM sodium phosphate pH 7.4 buffer, was used to remove excess DTT. To determine the second-order rate constants, 0.5 µM Hypocrates or 1 µM Hypocrates or Hyp was mixed with increased concentrations of an oxidant (NaOCl, NaNBr or N-chlorotaurine) in a reaction medium in 100 mM sodium phosphate buffer at 25 °C. Changes in fluorescence were monitored, and the obtained curves were fitted with double exponential equations which gave trustworthy diagnostic residual plots. From the double exponential equations, the observed apparent Michaelis–Menten parameters are summarized in Supplementary Table 4.

Gel filtration experiments. Gel filtration experiments were performed using a Superdex® 200 Increase 10/300 GL column (Cytiva) equilibrated with 100 mM sodium phosphate buffer (pH 7.4) at 2 °C and at a flow rate of 0.75 ml/min. The column was connected to an Agilent 1260 Inert LC system equipped with Agilent 1260 diode array detector and calibrated using cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (130 kDa), b-amylase (200 kDa) and ferritin (450 kDa). The equipment was controlled by Agilent OpenLAB. CDS Chemistry Data Analysis and RS3 software. Hypocrates samples were incubated in the presence of 5 mM DT or with N-chlorotaurine at 1:20 protein/oxidant ratio for 5 min before the injection (30 µl). The sensor was visualized by measuring absorbance at 415 nm to detect only molecules with the mature chromophore. The data were analyzed with OriginPro 9.0 (OriginLab).

Measurements of MPO activity with purified Hypocrates. To test whether Hypocrates is capable of visualizing MPO activity in vitro, the purified sensor was incubated with 0.1 U/ml human MPO and 100 µM H2O2 for 10 min in PBS, after the which the fluorescence excitation spectrum (λex = 525 nm) was recorded with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer controlled by Cary Eclipse Kinetics 1.132 Application. The probes that contained only MPO or H2O2 were treated according to the same protocol to control for non-specific fluorescence changes. The data were analyzed using OriginPro 9.0 (OriginLab).

To record the dynamics of HOCl production by MPO in vitro, purified Hypocrates was incubated in PBS in the presence of 0.1 U/ml human MPO, and the intensities of fluorescence (λem = 525 nm) excited at 425 and 500 nm were collected every 2.4 s with the use of a Varian Cary Eclipse Fluorescence Spectrophotometer controlled by Cary Eclipse Kinetics 1.132 Application. To start the MPO reaction, 100 µM H2O2 was added to the reaction mix. A probe without MPO was treated according to the same protocol to control for H2O2-induced fluorescence changes. A probe without MPO and without H2O2 addition was treated according to the same protocol to control for fluorescence changes attributed to prolonged incubation. For all three samples, the Exc/Em425 ratio was calculated as a function of time, and the first two curves were normalized to the third one. The data were analyzed using OriginPro 9.0 (OriginLab).

NaOCl visualization with Hypocrates, HypocratesCS, and SyPhet3s in HeLa Kyoio cells. HeLa Kyoio cells were cultured in DMEM (PanEko) supplemented with 10% FBS (Biosera), 2 mM L-glutamine (PanEko), 50 units/ml penicillin (PanEko) and 50 µg/ml streptomycin (PanEko) at 37 °C in an atmosphere containing 5% CO2. Cells were passaged every 2–3 days. For transfection, cells were seeded into 35-mm glass-bottom dishes (SPL Lifeiences). After 24 h, cells were transfected with the plasmid of the required sensor using FuGene HD transfection reagent (Promega) according to the manufacturer’s protocol. Confocal microscopy was performed on the next day after transfection with a Leica DMI 6000 microscope, equipped with an HCX PL Apo CS 40 × 1.25 Oil UV objective, CFP (excitation filter BP 436/20, dichromatic mirror 455, suppression filter BP 480/40) and YFP (excitation filter BP 515/60, dichromatic mirror 545, suppression filter BP 525/50) filter cubes. The microscope was controlled by LAS X 2.6.4.8702 software. A 10 mM stock solution of NaOCl (EMPLURA) in Milli-Q water was freshly prepared before cell imaging. Cell culture medium was replaced with 900 µl of PBS, and baseline fluorescence was detected for several minutes. PBS was chosen as an inorganic imaging medium because NaOCl, being a strong oxidant, can react with the nitrogen-containing components of the medium and thus introduce inaccuracy to the results. The desired amount of NaOCl stock was diluted in 100 µl of PBS just before addition, and the final concentration of NaOCl in the sample was in the range of 10–40 µM (~4.2–17 nmol/105 cells). All measurements were taken at room temperature because the maximum response amplitude of Hypocrates is being reduced as a result of heating. The responses of sensors were calculated as ratios of fluorescence intensities excited at 500 nm and at 425 nm (Exc/Em425 ratio) and normalized to the signal of the probe on the first image of the series. The processing of the images and quantification of results were performed using Fiji 2.0-rc-691.529.jar (https://fiji.sc), Excel 2016 (Microsoft), and Origin 2019 (OriginLab).

Polymorhonucleocytes isolation. Polymorhonucleocytes (PMNs), including neutrophils, were isolated from the whole blood of healthy volunteers. All donors were informed of the final use of their blood and signed an informed consent document. The study was approved by the local ethics committee of Pirogov Russian National Research Medical University and conducted in accordance with the Declaration of Helsinki. The blood samples were collected in tubes with anticoagulant (EDTA) and separated on Polymorpher® (Alere Technologies AS) gradient according to the manufacturer’s protocol. After blood separation by
centrifugation, the layers containing serum and PMNs were transferred to indi-vidual tubes. The collected serum was further used for the osmopsonization of bacteria. PMNs were further washed twice with PBS by centrifugation (400 × g, 10 min) and resuspended in RPMI-1640 medium (PanEko) supplemented with 2 mM l-glutamine (PanEko) and 0.5% FBS (Biosera). The concentration of the PMNs was adjusted to 1.5 × 10⁶ cells/ml. For activation, the PMNs were incubated with 1 µg/ml recombinant human IFN-γ (GibcoTM) for 1.5 h in the CO₂ incubator (37 °C, 5% CO₂). Then, the liquid was replaced with the same medium without IFN-γ by centrifugation and cells were kept in the CO₂ incubator (37 °C, 5% CO₂) until the imaging experiments.

**Live-cell imaging of phagocytosis.** To obtain bacterial cells expressing Hypocrates or HypocratesCS, chemically competent E. coli XL1-Blue cells were transformed with the pQE30 vectors encoding the required constructs. The bacterial colonies were grown on LB-agar plates with ampicillin (150 µg/ml) for 14 h at 37 °C. To achieve better protein maturation, the plates were additionally kept at room temperature for two more days. For opsonization, the bacteria were diluted in 1 ml of PBS and human serum mixture (1:1), after which the sample was incubated in a thermoshaker for 30 min at 37 °C, 300–400 rpm. Opsinorized bacteria were washed three times with PBS by centrifugation (1200g, 2 min) and the collected cellular mass was resuspended in the same buffer to obtain an OD₆₀₀ of 0.5.

For live-cell imaging of the PMNs, the medium was changed to RPMI-1640 without phenol red and sodium bicarbonate (Sigma-Aldrich) supplemented with 2 mM l-glutamine (Sigma-Aldrich), 0.5% FBS (Biosera), and 20 mM HEPES. The procedure was centrifuged at 400g for 10 min. At the next step, 800 µl of the PMNs suspension (1.8 × 10⁶ cells/ml) were transferred into a 35 mm confocal dish (SPL Lifesciences) for microscopy. To induce phagocytosis, 75 µl of opsonized E. coli cells in PBS (OD₆₀₀ = 0.5) were added per dish. Microscopy was performed with an ECLIPSE Ti2 inverted microscope (Nikon Instruments Inc.) equipped with Plan Apo VC 100X NA 1.40 oil objective. The microscope was controlled by NIS-Elements 5.21.03 software. The signal was detected in three channels: 395 nm diode (exposure 100 ms, diode intensity 9%), 470 nm diode (exposure 200 ms, diode intensity 9%), and brightfield. The signals of Hypocrates and HypocratesCS in bacteria were calculated as ratios of the fluorescent intensities excited at 470 nm and 395 nm (Ex₄70/Ex₃95). To measure the response amplitudes, the signals were normalized to the values observed before the onset of phagocytosis. Image processing and quantification of results were performed using Fiji 2.0.0-rc-69/L52pJava 1.8.0_172 (https://fiji.sc). Excel 2016 (Microsoft), and OriginPro 9.0 (OriginLab).

**Danio rerio tail fin inflammation model.** The study was approved by French Ministry of Agriculture (n°C75-05-12). For the tail fin amputation experiment, mRNAs of Hypocrates, HypocratesCS, and HyPerRed were in vitro synthesized using the mMessage mMachine Transcription Kit (Invitrogen) according to manufacturer’s manual. For transient expression of the biosensors in zebrafish larvae, a single type Tübingen (TU) strain, male and female, 80 ng/ml of Hypocrates or HypocratesCS mRNA and 50 ng/µl of HyPerRed mRNA were co-injected into 1-cell-stage embryos. The zebrafish embryos were maintained in egg water containing 0.2 mM N-phenylthiourea (PTU; Sigma) to prevent pigment formation at 28 °C. Fluorescence imaging was performed 48 h post-fertilization (hpf). Larvae were anesthetized in 0.02% MS-222 tricaine (Sigma), embedded in low-melting agarose (0.8%) and then subjected to tail centrifugation before amputation. A statistical two-way ANOVA test with a Bonferroni post hoc defined the significance of the results. For transient expression of the biosensors in zebrafish tail, cell-stage embryos. The zebrafish larvae (wild-type Tübingen (TU) strain, male and female), 80 ng/µl of Hypocrates or HypocratesCS, 2m ML-glutamine (Sigma-Aldrich), 0.5% FBS (Biosera), and 20 mM HEPES. This liquid was replaced with the same medium without IFN-γ by centrifugation and the fluorescence imaging was performed 48 h post-fertilization (hpf). Larvae were anesthetized in 0.02% MS-222 tricaine (Sigma), embedded in low-melting agarose (0.8%) and then subjected to tail centrifugation before amputation. A statistical two-way ANOVA test with a Bonferroni post hoc defined the significance of the results.
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Author contributions
A.L.K. developed architecture and design of the working version of Hypocrates biosensor, performed the in vitro experiments, analyzed and combined data, and wrote the manuscript. M.-A.T. crystallized, collected data, and solved the structure of HypocratesCS, performed the in vitro circular dichroism experiments, pKa determination, fluorescence selectivity and sensitivity tests, pre-steady-state kinetic measurements of Hyp8 and NemR, prepared Hypocrates MS samples with different oxidizing agents, analyzed data, and wrote the manuscript. A.S.P. performed experiments in eukaryotic cell culture. M.T. performed experiments in zebrafish. R.I.R. performed the in vitro experiments and analyzed the data. D.E. performed selectivity and kinase assays, analyzed the data, and edited the manuscript. K.W. helped with the crystal conditions optimization and performed kinetics experiments.
measurements. I.V.M. helped with X-ray structure refinement, and X-ray data deposition. A.D.S. performed experiments with neutrophils. D.V. collected the mass spectrometry data and analyzed the MS data. A.Yu.G. performed gel filtration experiments. M.S.B. synthesized chemical compounds (NCT, NaOBr, ONOO’). S.V. supervised the in vivo work. J.M. supervised the in vitro kinetic and structural studies, analyzed data, and wrote the manuscript. D.S.B. and V.V.B. created the general concept of the project, supervised the work of the project at all stages, wrote the manuscript.

**Competing interests**
The authors declare no competing interests.

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