Chlorine Dioxide Is a Size-Selective Antimicrobial Agent

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Abstract

Background / Aims: ClO₂, the so-called “ideal biocide”, could also be applied as an antiseptic if it was understood why the solution killing microbes rapidly does not cause any harm to humans or to animals. Our aim was to find the source of that selectivity by studying its reaction-diffusion mechanism both theoretically and experimentally.

Methods: ClO₂ permeation measurements through protein membranes were performed and the time delay of ClO₂ transport due to reaction and diffusion was determined. To calculate ClO₂ penetration depths and estimate bacterial killing times, approximate solutions of the reaction-diffusion equation were derived. In these calculations evaporation rates of ClO₂ were also measured and taken into account.

Results: The rate law of the reaction-diffusion model predicts that the killing time is proportional to the square of the characteristic size (e.g. diameter) of a body, thus, small ones will be killed extremely fast. For example, the killing time for a bacterium is on the order of milliseconds in a 300 ppm ClO₂ solution. Thus, a few minutes of contact time (limited by the volatility of ClO₂) is quite enough to kill all bacteria, but short enough to keep ClO₂ penetration into the living tissues of a greater organism safely below 0.1 mm, minimizing cytotoxic effects when applying it as an antiseptic. Additional properties of ClO₂, advantageous for an antiseptic, are also discussed. Most importantly, that bacteria are not able to develop resistance against ClO₂ as it reacts with biological thiols which play a vital role in all living organisms.

Conclusion: Selectivity of ClO₂ between humans and bacteria is based not on their different biochemistry, but on their different size. We hope initiating clinical applications of this promising local antiseptic.

Introduction

The emergence and dissemination of new antibiotic-resistant bacterial strains caused by an overuse of antibiotics [1] is a global public-health concern. Methicillin Resistant Staphylococcus aureus (MRSA) [1,2] and Carbapenem- or Extreme Drug-Resistant Acinetobacter baumannii [3,4] are only two well known examples for such bacteria attracting world wide attention. Moreover, while the number of antibiotic resistant infections is on the rise, the number of new antibiotics is declining [1,2]. As a result of such a dangerous situation, searches for new antimicrobial agents, as well as strategies including a switch from antibiotic to antiseptic therapies, whenever that is feasible, have been initiated.

When treating local infections of wounds, ulcers or an infected mucous membrane, the application of antiseptics instead of antibiotics is a reasonable alternative especially because bacteria are less able to develop resistance against them [5]. Presently the majority of the antiseptics used for wounds [6] are organic compounds. The most frequently applied ones [6] are chlorhexidine (chlorhexidine digluconate), octenidine (octanidine dihydrochloride), polyhexanide (polyhexamethylene biguanide) and triclosan (5-chlorine-2-(2,4-dichlorophenoxy)-phenol). Notable exceptions are PVP-iodine (poly(vinylpyrrolidone)-iodine complex) [6] where the active ingredient is iodine, and silver [7], both being inorganic compounds.
There are some other, less used, inorganic antiseptics such as aqueous sodium hypochlorite (NaOCl), or hydrogen peroxide (H₂O₂) solutions, or ozone (O₃) gas which have some applications in dentistry [8]. These compounds, however, are mainly used as disinfectants because they can be toxic even in low concentrations, a property seriously limiting their antiseptic applications. NaOCl, for example, one of the most commonly used components of irrigating solutions in endodontic practice, can cause poisoning and extensive tissue destruction if it is injected (inadvertently) into periapical tissues in the course of endodontic therapy [9]. H₂O₂ is also a double edged sword against bacteria as it also hurts living tissue [10]. Moreover, many bacteria are able to resist H₂O₂, as their catalase enzyme is able to decompose H₂O₂ rapidly [11]. Thus, beside toxicity, resistance can be also a problem even with the use of inorganic disinfectants [5]. It would be therefore reasonable to choose an antiseptic which would be free of such problems. We believe that in this respect chlorine dioxide (ClO₂) may be the right choice, moreover ClO₂ has other characteristic features favourable for antiseptic applications.

In the last twenty or more years chlorine dioxide emerged as a new and popular inorganic disinfectant. It is often referred to as „the ideal biocide” [12] because of its advantageous properties. In spite of that, as far as we know, ClO₂ solutions are not frequently used as antiseptic. This is because the available ClO₂ solutions were more or less contaminated with other chemicals applied in its synthesis and that contamination formed a major obstacle in medical applications like treating infected wounds, for example. Since 2006, however, with the help of an invention [13], it is relatively easy to produce high purity aqueous ClO₂ solutions. These solutions are already commercially available [14] and have been successfully used in dentistry [15] since 2008. Thus, it seems reasonable to ask the question whether the “ideal biocide” in its pure form can also be an “ideal local antiseptic” at the same time.

Such an ideal local antiseptic should satisfy many criteria. First of all, it should be safe: it should act only locally to avoid the danger of systemic poisoning and should not inflict cytotoxic effects even in the disinfected area. In this respect, it is one of the main aims of the present work to find a reasonable answer for the following intriguing question: how is it possible that contacting or even drinking ClO₂ solution is practically harmless for animals [16] and human beings [17], while the same aqueous solution can be a very effective and a rapid killer for bacteria, fungi, and viruses? What is the basis of this unexpected selectivity?

The answer suggested in the Results section is the following: the selectivity between humans or animals and microbes is based not on their different biochemistry, but on their different size. Denominating ClO₂ in the title as a „size selective” antimicrobial agent aims to emphasize this new type of selectivity. To reach that conclusion, ClO₂ transport was studied experimentally via protein membranes. The results of these experiments were evaluated applying a reaction-diffusion model for the ClO₂ transport in a reactive medium to obtain the diffusion coefficient of ClO₂, and the concentration of reactive groups in a protein medium. Based on these parameters the killing time, the time needed to flood a bacterium completely with ClO₂, can be calculated. (Details of the reaction-diffusion model and the derivation of formulae estimating the killing time are given in the Information S1.) It was found that the characteristic time necessary to kill a microbe is only a few milliseconds. As ClO₂ is a rather volatile compound its contact time (its staying on the treated surface) is limited to a few minutes. While this stay is safely long enough (being at least 3 orders of magnitude longer than the killing time) to inactivate all bacteria on the surface of the organism, it is too short for ClO₂ to penetrate deeper than few tenths of a millimetre; thus, it cannot cause any real harm to an organism which is much larger than a bacterium.

In the Discussion part, it is shown that ClO₂ can meet the safety and effectiveness requirements for a local antiseptic. Next, the chemical mechanism of the antiseptic action of ClO₂ is discussed and compared with that of hypochlorous and hypiodous acids (HOCI and HOI) which are „natural” antiseptics. These hypohalous acids are used by neutrophil granulocytes, the most abundant type of white blood cells in mammals, to kill bacteria after phagocytosis. Both hypohalous acids and also ClO₂ attack sulfhydryl groups [18,19] which play an essential role in the life processes of all living systems, e.g. in ATP synthesis. That explains why bacteria were not able to develop resistance against HOCI during eons of evolution and why the emergence of ClO₂ resistant bacterial strains cannot be expected either. Besides this similarity, however, there are also important dissimilarities among these reagents, e.g. ClO₂ is more selective than HOCI. Last of all, circulation in multicellular organisms can provide some additional protection to these organisms against ClO₂.

**Methods**

**Materials**

Reagent grade chemicals were purchased from Sigma-Aldrich and pork skin gelatine from Fluka (48719). High purity chlorine dioxide solutions were produced according to our invention [13]. Dried pig bladders were purchased in the Great Market Hall of Budapest at the shop “Solvent” (www.solvent.hu). These bladders are usually applied for kulen sausage production.

**Physico-chemical methods**

**Measurement of ClO₂ permeation through protein membranes.** The rate of ClO₂ transport was measured with the apparatus shown in Figure 1 through two kinds of protein membranes: gelatin and pig bladder membranes, respectively. Choosing a membrane geometry for the experiments is advantageous because then the problem is „one dimensional”, the concentration is a function of only one spatial coordinate x, which is perpendicular to the membrane, and the concentration distribution can be given as \( c=c(x,t) \).

As Figure 1 shows, the membrane is in a horizontal position and the transport of ClO₂ takes place across the membrane bounded by two horizontal planes we denote by \( x=0 \) and \( x=d \) in our calculations, where \( d \) is the thickness of the membrane.

Constant ClO₂ concentrations are maintained at both boundaries of the membrane, i.e. we have constant boundary conditions for the con...
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![Diagram of apparatus to measure ClO₂ transport through gelatine or pig bladder membranes](image)

Figure 1. Apparatus to measure ClO₂ transport through gelatine or pig bladder membranes. The two glass parts of the apparatus are held together by a pair of extension clamps (not shown in the Figure) which are fixed to a support stand by clamp holders. The active cross-section of the membranes is 28 cm². See text for the working principle.

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Initial conditions: $c(0,t) = c_0$, and $c(d,t) = 0$, respectively. There is no ClO₂ in the membrane at the start of the experiment, so the initial condition: $c(0<x<d,0) = 0$ (see Figure S1 in Information S1).

While the lower face of the protein membrane is not in direct contact with the liquid phase, such direct contact would not make any difference regarding the ClO₂ transport. This is because the chemical potential of ClO₂ in the liquid and the vapour phase is the same due to the equilibrium between the liquid and the vapour phase established by continuous stirring.

Above the protein membrane there is a silicone rubber membrane (permeable for ClO₂ only) that is reinforced by filter paper and the gelatin was cross-linked with glutaraldehyde. As the cellulose in the filter paper does not react with ClO₂ from the point of our experiments, it is an inert material.

Both protein membranes had a thickness of 0.5 mm and a diameter of 10 cm. The diameter of the active area in the apparatus was 6 cm resulting in an active area of 28 cm². The volume of the aqueous ClO₂ solution was 40 ml and its ClO₂ concentration was around 1000 ppm. (The exact value is given at each experiment.)

After crossing the membranes, ClO₂ enters the upper aqueous solution which is made by mixing 10 ml of water, 2 ml of 1 M sulphuric acid, 1 ml of 1 M KI, and 0.5 ml of 0.01 M Na₂S₂O₃ and as an indicator, two drops of 5 % starch solution is also added. When ClO₂ enters the upper solution, it oxidizes iodide to iodine, which, in turn, is reduced back to iodide again by Na₂S₂O₃ as long as thiosulphate is in excess. However, when all thiosulphate is consumed, the intense blue-black colour of the starch-triiodide complex appears suddenly. The time $t$ when the whole solution becomes homogeneously black (the time of the „black burst”) was recorded and another 0.5 ml of Na₂S₂O₃ solution was added with the help of the syringe shown in the Figure. Addition of the thiosulphate eliminated the blue-black colour immediately but, after a certain period, when enough new ClO₂ was transported across the membrane, it reappeared again. Then the cycle was repeated starting with the injection of a new 0.5 ml portion of the Na₂S₂O₃ solution. The results of the measurements were depicted in a $V=V(t)$ diagram where $t$ is the time of the $n$-th dark burst and $V = n \times 0.5$ ml that is the total volume of the thiosulphate solution added before the $n$-th breakthrough.

The experiments were performed at laboratory temperature $24 \pm 2 \, ^\circ C$.

Preparation of the gelatin membrane

To prepare a mechanically strong membrane, it was reinforced by filter paper and the gelatin was cross-linked with glutaraldehyde. As the cellulose in the filter paper does not react with ClO₂ from the point of our experiments, it is an inert material.

10 ml of 10 % aqueous gelatin solution was mixed rapidly with 0.5 ml of 25 % glutaraldehyde solution at room temperature, and a filter paper disk (diameter: 10 cm) was soaked with the mixture. Then the disk was placed between two glass plates covered with polyethylene foils. Spacers were applied to produce a 0.5 mm thick membrane. After a 2 hour setting time the filter paper reinforced gelatin membrane was removed from the form and it was placed into distilled water overnight before the measurements.

Preparation of the pig bladder membrane

For the experiments, membrane disks with 10 cm diameters were cut from commercially available pig bladders and they were kept in distilled water for one day at +4 °C to stabilize their water content. The pig bladder membranes are slightly asymmetric: the surface of one side is smoother than the other. To obtain reproducible results, the membrane was always fixed in the apparatus with its smoother side facing downwards.

Results

Our results cover the following themes: First, we present and evaluate membrane transport experiments aiming to determine

i) the diffusion coefficient of ClO₂ D in a reactive protein medium, and

ii) the concentration of reactive groups $s_0$ in that medium.

To evaluate the membrane transport experiments we applied a reaction-diffusion model for the transport of ClO₂ in a medium containing reactive proteins. The details of that theory and the
mathematical derivation of formulas applied in this section are
given in the Information S1. Then, based on the experimentally
determined $D$ and $s$, we calculate $T_{kill}$, the time needed to kill
bacteria by ClO$_2$, and $p$, the penetration depth of ClO$_2$ into
human tissue during a wound healing treatment.

ClO$_2$ permeation was measured via gelatin and pig bladder
membranes. The apparatus is shown in Figure 1 of the
Methods section.

Permeation of ClO$_2$ through an artificial gelatin membrane

Gelatin was our first choice for a model material because we
wanted to study the ClO$_2$ transport in a protein medium with a
known amino acid composition. Pork skin gelatin (Fluka 48719)
contains only two amino acids that can react with ClO$_2$: methionine (0.88 %) and tyrosine (0.6 %) [20].

Figure 2 shows the results of two consecutive experiments
performed with the same gelatin membrane (see the two
curves denoted as 1st exp. and 2nd exp.). After the first
experiment, the membrane was removed from the apparatus
and was kept in distilled water for 1 hour before the second experiment.

Calculating the ClO$_2$ diffusion coefficient $D$ and the
effective concentration of ClO$_2$ consuming substrates
$s_e$ in gelatin

Figure 2 shows N, the cumulated amount of ClO2 permeated
through the membrane as a function of time.

(N was calculated from the titrant volumes V that are given in
Table S1 in Information S1. together with the times t of
addition.) It is a common feature of both curves shown in
Figure 2 that two characteristically different dynamical regimes
can be observed. In the first regime, the amount of the
permeated ClO2 is very small, then, after a rapid transition
period the cumulated amount of ClO2 increases linearly with
time. Real dynamics can be approximated with the following
simplified model: zero permeation is assumed at the beginning
during a waiting period but right after that a constant diffusion
current appears, thus, the permeated amount increases linearly
with time. To characterize such a dynamic behaviour the
concept of “time lag” can be introduced: it is the time where the
asymptote of the linear regime crosses the time axis [21].

Regarding the asymptotes of the corresponding curves, the
time lag in the first and in the second experiment is $T_{L1} = 627$ s
and $T_{L2} = 175$ s, respectively. A logical explanation for this
difference is that some ClO$_2$ is consumed inside the gelatin in
the rapid reaction with methionine and tyrosine. So ClO$_2$ can
breakthrough only after it eliminates all these highly reactive
amino acid residues. In the case of the second experiment, the
breakthrough occurs earlier as most of these residues already
reacted with ClO$_2$ during the first experiment.

If we assume that in the second experiment the reaction
plays a minor role only, then in that case, the time lag is
due to diffusion. Roughly speaking the diffusional time
lag is the time necessary to establish a steady state
centration profile inside the membrane that is to “fill up” the
membrane with ClO$_2$. Based on dimensional analysis
considerations (the dimension of the diffusion coefficient is
(length)$^2$/ (time) ) we can expect that the time lag should be
proportional with the square of the thickness and inversely
proportional with the diffusional coefficient. Really, the exact
result [21] is that the diffusional time lag $T_{DM}$ for a membrane of
thickness $d$ can be calculated as:

$$ T_{DM} = \frac{1}{6} \cdot \frac{d^2}{D} \quad (1) $$

Thus, with the assumption $T_{L2} = T_{DM} = 175$ s, $D$, the diffusion
coefficient of ClO$_2$ in the gelatin membrane can be calculated
knowing that $d = 0.5$ mm. The result: $D = 2.4 \times 10^{-6}$ cm$^2$s$^{-1}$.

$D$ can be determined in another way as well, from the steady
state regime. The steady state ClO$_2$ current is the slope of the
curve in the linear regime. For the 2nd experiment $J_2 = 30$
nmol/s. Then Fick’s law of diffusion

$$ J = A \cdot D \cdot \frac{\Delta c}{\Delta t} \quad (2) $$

can be applied to calculate $D$. Here $A= 28.3$ cm$^2$ is the active
cross-section of the membrane and $\Delta c$ is the concentration
difference between the two sides of the membrane. Regarding
our boundary conditions $\Delta c = c_0 = 20.1 \times 10^{-3}$ M. This way $D = 2.6 \times 10^{-6}$ cm$^2$s$^{-1}$ is obtained.

The two $D$ values, the one calculated from the time lag and
the other calculated from the steady state, agree reasonably
well indicating that indeed the 175 s time lag is caused mostly
by diffusion and any delay due to chemical reactions is
negligible in the second experiment.

On the other hand, in the first experiment, the time lag $T_{DM}$
is caused mostly by the reaction between ClO$_2$ and the reactive
amino acid residues (in short “substrates”) in the membrane. It
is important to realize that $T_{DM}$ is not due to a slowness of the
reaction kinetics (as the rate constants of the relevant ClO₂– amino acid reactions are relatively high [22–24]), but it is due to the actual ClO₂ consumption by the reactions within the membrane delaying the breakthrough. If we assume that the rate of the chemical reaction is limited by the diffusional transport of ClO₂ across a zone already without reactive amino acids toward a zone of unreacted ones, then a sharp reaction front will develop on the boundary of the two zones (see Figure S1 in Information S1). The front starting from one side of the membrane and driven by diffusion, propagates slowly through the membrane and \( T_{RM} \) is the time when it arrives to the other side of the membrane. According to a detailed derivation in the Information S1, \( T_{RM} \) can be given by the so-called parabolic rate law (see equation (S12) in Information S1):

\[
T_{RM} = \frac{1}{2} \frac{s_0}{c_0} \cdot \frac{d^2}{D}
\]

(3)

where \( s_0 \) is the initial effective substrate concentration, i.e. the ClO₂ consuming capacity of the membrane in unit volume, and \( c_0 \) is ClO₂ concentration at the boundary of the membrane.

Substituting the more reliable diffusion coefficient measured in the steady-state of the second experiment \( D = 2.6 \times 10^{-6} \text{ cm}^2\text{s}^{-1} \) and applying the assumption that \( T_{RM} = T_{1,1} = 627 \text{ s} \), the effective substrate concentration of the gelatin membrane \( s_0 \) can be calculated. The result: \( s_0 = 26.2 \text{ mM} \).

**Permeation of ClO₂ through a pig bladder membrane**

In this experiment, we studied the ClO₂ permeability of a pig bladder membrane which is a relatively thin (in our case it was 0.5 mm thick) but sturdy animal tissue. The same apparatus was applied as in the case of the gelatin membrane and the experimental points were depicted in Figure 3 also with the same method. (Titrant volumes and the time of addition are given in Table S2 in Information S1.)

All the three measurements (indicated as 1st day, 2nd day and 3rd day) were performed with the same pig bladder membrane but on three successive days. The membrane was kept in distilled water at +4 °C overnight between the experiments which were always started with fresh solutions.

To check the reproducibility of our measurements, we repeated the measurements with another pig bladder membrane (not shown in the Figure). While the new membrane was from a different pig bladder and its blood vessel pattern was also different, the relative deviation between the results of the two series of experiments was surprisingly small: only about 10 %. (The blood vessel structure of the membrane becomes visible as a dark network before a „black burst” because the permeability of the membrane is somewhat higher through those vessels.)

Another interesting observation was that the pig bladder membrane maintained its integrity and its mechanical strength even after the third experiment. This is because ClO₂ reacts selectively with certain amino acid residues of the proteins but does not destroy the peptide bonds thus the primary protein structure can survive.

**Calculating the ClO₂ diffusion coefficient and the effective concentration of ClO₂ consuming substrates in pig bladder**

Evaluation of the results was made in a similar way as in the case of the gelatin membrane. It was assumed that the time lag measured in the third experiment \( T_{1,3} = 226 \text{ s} \) is a purely diffusional time lag that is \( T_{1,3} \approx T_{RM} \). The diffusion coefficient of ClO₂ in a pig bladder membrane calculated from the above assumption is \( D = 1.84 \times 10^{-6} \text{ cm}^2\text{s}^{-1} \). That value is in good agreement with the \( D = 1.80 \times 10^{-6} \text{ cm}^2\text{s}^{-1} \) value calculated from the steady state current \( J = 14.1 \text{ nmol/s} \) of the 3rd experiment.

As we can see, the diffusion coefficient of ClO₂ in a pig bladder tissue is only 30 % smaller than in the unstructured gelatin. This supports our assumption that the cellular structure of the pig bladder tissue does not matter too much from the point of the diffusional transport of ClO₂ as it can penetrate through the external and internal lipid membranes of the individual cells of the tissue.

However, there is a more significant deviation between the pig bladder and the gelatin regarding \( s_0 \), the effective substrate concentration. Assuming that the time lag in the first experiment \( T_{1,1} = 2770 \text{ s} \) is due to the chemical reaction, \( T_{1,1} \approx T_{RM} \), then from (3) we get \( s_0 = 56 \text{ mM} \), indicating that the concentration of the reactive components in the pig bladder tissue is about two times higher than that is in the gelatin. This is a reasonable result as the animal tissue is denser, and it contains not only methionine and tyrosine like gelatin, but also cysteine and tryptophan residues.

We would like to add that in a series of measurements performed with the same membrane the steady state ClO₂ current in the first experiment is always smaller than in the subsequent ones, although the ClO₂ source is not changed. This effect is more pronounced in the case of an animal membrane (compare the slope of the 1st day experiment with that of the other days). The phenomenon can be understood if we assume that some components, which are able to react
with ClO₂ but only slowly, can remain in the pig bladder even after the first ClO₂ breakthrough. As it is shown in equation (S40) in the Information S1, the slow ClO₂ consumption of these components can explain a smaller quasi-steady state current. The fact that these components disappear from the membrane after keeping it in water overnight suggests that they are reaction products which can be leached out from the membrane or are unstable intermediates which decompose.

**Estimating the killing time for bacteria with cylindrical and spherical geometries**

We assume that a bacterium is killed when its whole volume is flooded by ClO₂. To calculate the killing time, if we know the shape and the size of the bacterium, we would need two more parameters, the diffusion coefficient of ClO₂, D and the effective concentration of ClO₂ consuming substrates, \( s_0 \), in the bacterial medium. In the absence of bacterial data it will be assumed that the parameters \( D \) and \( s_0 \) in the single cell of a bacterium are close to that what we have measured above in the animal cell aggregates of the pig bladder. A further simplifying assumption is that only spherical and cylindrical bacteria are considered. Numerical results are calculated for a diameter of 1 μm, which is a characteristic length-scale for bacteria. Mathematical formulas for the killing time and the penetration depth are derived in the Information S1. In this section only the results of those derivations will be given together with some qualitative explanations on their meaning.

It will be assumed that the rate of the “ClO₂ – bacterium reaction” is also limited by the diffusion of ClO₂ to the fast reacting amino acid residues fixed in protein molecules like in the case of the much larger membranes and this way a sharp reaction front propagates from the cell wall toward the centre of the bacterium.

Intuitively, the killing time \( T_{KILL} \) should be analogous to the time lag \( T_{BL} \) in a membrane caused by a chemical reaction, because these are the times needed to flood the whole volume. We can expect, however, that the geometric factor should be different depending on the shape of the bacterium. For a cylindrical bacterium with a diameter of \( d \) the killing time is

\[
T_{KILL,C} = \frac{1}{16} \cdot \frac{s_0}{c_0} \cdot \frac{d^2}{D}
\]

(4)

see equation (S18) in the Information S1, and for a spherical bacterium also with a diameter of \( d \) it is

\[
T_{KILL,S} = \frac{1}{24} \cdot \frac{s_0}{c_0} \cdot \frac{d^2}{D}
\]

(5)

according to equation (S24) in Information S1. We can see that (4) and (5) are analogous to (3) but the geometric factors for a cylinder and for a sphere are much smaller than for the planar membrane indicating that in these geometries the surface from where diffusion current is starting is relatively larger compared to the volume that has to be flooded.

Substituting the pig bladder parameters \( D = 1.8 \times 10^{-6} \text{ cm}^2 \text{s}^{-1} \) and \( s_0 = 56 \text{ mM} \) into formulas (4,5) together with the ClO₂ concentration applied in the wound healing experiments (see later) \( c_0 = 4.45 \text{ mM} \) (Solumium Oral®, 300 ppm) and using \( d = 1 \mu \text{m} \) we obtain that the killing time for a cylindrical bacterium with a diameter of 1 μm is

\[
T_{KILL,C} = 4.4 \text{ ms},
\]

while the killing time for a spherical bacterium with a diameter of 1 μm is

\[
T_{KILL,S} = 2.9 \text{ ms}.
\]

As we can see, the killing time for a bacterium is only a few ms due to its small size. Even if \( s_0 \), the effective substrate concentration of a bacterium would be an order of magnitude higher than what we assumed, the killing time would be still less than 0.1 s. Other approximations applied in our calculations can only overestimate the real killing time. For example, the diffusion coefficient of ClO₂ in the pig bladder was measured at 24 ± 2 °C. If ClO₂ is used to disinfect a living human tissue, the temperature is higher, which means a larger diffusion coefficient and an even shorter killing time. Another approximation is the concept of fixed substrates. Inside a bacterium mobile substrates like glutathione [25], free amino acids and various antioxidants also occur. These small molecules can diffuse by and large freely within the bacterium. Nevertheless \( T_{KILL} \) would still work as a good upper estimate because the mobility of the substrate can only shorten the time needed for ClO₂ to reach these substrates and react with them. Furthermore, when the killing time \( T_{KILL} \) is regarded as the time when the sharp front reaches the center of the sphere or the symmetry axis of the cylindrical bacterium, it will surely be overestimated, as it is not necessary to oxidize all the available substrate content of a bacterium to kill it. For example, it is enough to oxidize less than 40 % of the methionine content of *E. coli* to achieve a 100 % kill [26].

**Contact time and penetration depth of ClO₂ into human skin or wound**

When an organism is not submerged in the aqueous ClO₂ solution but the solution is applied on its surface only, as in the case of disinfecting wounds, the volatility of ClO₂ also has to be taken into account. The effective contact time is much shorter using a ClO₂ solution than with less or non-volatile disinfectants. According to our measurements, when a wound is covered with 3 wet and 3 dry layers of gauze more than 80 % of ClO₂ evaporates from the bandage within one minute due to the high volatility of ClO₂ and to the high specific surface of the gauze. Thus, to give an upper limit for the penetration depth into the human tissue, we will assume that the initial ClO₂ concentration \( c_0 = 4.45 \text{ mM} \) (Solumium Oral®) is maintained for 60 s, that is \( T_{CON} = 60 \text{ s} \), where \( T_{CON} \) denotes the contact time. As a zero-th estimate, we assume again that the human tissue has the same \( D \) and \( s_0 \) values like that of the pig bladder tissue.

Applying the parabolic rate law (see equation (S13) in Information S1 where \( t = T_{CON} \) the penetration depth \( p \) can be estimated:

\[
p = \sqrt{\frac{2 c_0 D \cdot T_{CON}}{s_0}}
\]

(6)
that layer should be much lower compared to the underlying
the capillary circulation which is present in living tissue but is
absent from dead tissue like the pig bladder membrane used
for the measurements. The serum in the blood vessels and also
the extracellular fluid contain many components capable
of reacting rapidly with ClO\textsubscript{2}. The fluid transport of these
reactive components in the blood capillaries of the dermis [27]
can maintain a finite reactant concentration in that region. Then
the diffusive transport of these reactants outward from the
dermis into the epidermis [27] can halt an inward propagating
reaction front establishing a steady state.

Moreover, in the case of intact human skin, ClO\textsubscript{2} should
permeate through the stratum corneum [28] first, which is the
10–40 μm thick outermost layer of epidermis consisting of
several layers of dead cells. This keratinous layer forms a
barrier to protect the underlying tissue from infection,
dehydration and chemicals. The diffusion coefficient of ClO\textsubscript{2}
in that layer should be much lower compared to the underlying
tissue.

As we can see, the penetration depth into human skin is only
few tens of a micrometer even if we neglect circulation. Such
shallow penetration cannot really harm human tissues. On the
other hand, this short contact time is still several orders of
magnitude larger than the killing time, \(T_{\text{CON}} >> T_{\text{KILL}}\), which
is the necessary criterion of a successful disinfection.

### Therapeutic window

The above formulas and calculations indicate that
disinfection of living tissues with aqueous ClO\textsubscript{2} solutions has a
very wide therapeutic window: while surprisingly low
concentrations and short contact times are able to kill bacteria,
much higher concentrations and residence times are still safe
to use.

There is one notable exception: inhaling high concentration
ClO\textsubscript{2} gases for an extended time can be dangerous for human
health because the alveolar membrane is extremely thin (a
mere 1-2 microns and in some places even below 1 micron).
The effect of ClO\textsubscript{2} in these membranes is somewhat
counterbalanced, however, by the intense blood circulation
there.

### Discussion

In this section first we discuss whether ClO\textsubscript{2} should be
regarded as an "exotic" antiseptic only or it has the promise to
become a commonly used antiseptic to treat local infections.
To this end safety and effectiveness requirements for a local
antiseptic are collected to check how ClO\textsubscript{2} can meet these
requirements compared to other antiseptics.

Next a biochemical action mechanism, explaining the
antiseptic effect of ClO\textsubscript{2}, is discussed, which is partly analogous
to that of hypochlorous and hypiodous acids. These "natural"
antiseptics also react, among others, with sulfhydryl groups like
ClO\textsubscript{2} but their reaction products can be different. The
importance of that difference and the protective role of SH
groups and of the circulatory system, existing in a multicellular
organism only, is also discussed.

### Safety and effectiveness requirements for a local
antiseptic

A local antiseptic should meet the following requirements to
be considered as safe:

i) it should act only locally to avoid systemic poisoning, and

ii) it should not prevent or delay the process of healing, i.e. it
should not be cytotoxic.

and as effective:

iii) it should be effectual in relatively low concentrations, and

even in biofilms (biofilms are medically important, accounting
for over 80 percent of microbial infections in the body [29]) as
well, and

iv) microbes should not be able to develop resistance against
it (a problem related to the biochemical mechanism of action).

As it was shown in the Results section ClO\textsubscript{2} as a size
selective antiseptic, meets requirements i) and ii). Thus only
criteria iii) and iv) are discussed here.

### Comparing the biocidal activity of ClO\textsubscript{2} to that of other
antiseptics (criterion iii)

In free aqueous solutions, the strongest chemical disinfectant
is ozone. In biofilms, however, the performance of ozone is
rather poor. In addition, ozone is toxic and decomposes in
aqueous solutions rapidly. (Its half life is only 15 min at 25 °C at
pH 7.) All of these disadvantageous properties of ozone
prevent its use as an antiseptic in most applications.

The second strongest disinfectant after ozone is chlorine
dioxide. Tanner [30] made a comparative testing of eleven
disinfectants on three test organisms (including two bacteria:
Staphylococcus aureus and Pseudomonas aeruginosa and one
yeast: Saccharomyces cerevisiae). He found that the
disinfectant containing ClO\textsubscript{2} had the highest biocidal activity on
a mg/l basis against the test organisms. Beside antibacterial
and antifungal properties, ClO\textsubscript{2} also shows strong antiviral
activity, about ten times higher than that of sodium hypochlorite
[31]. And it inactivates practically all microbes including algae
and animal planktons [32] and protozoans [33].

Moreover ClO\textsubscript{2} can remove biofilms swiftly [12] because it is
highly soluble in water and unlike ozone it does not react with
the extracellular polysaccharides of the biofilm. This way ClO\textsubscript{2}
can penetrate into biofilms rapidly to reach and kill the
microbes living within the film.

### Impossibility of bacterial resistance against ClO\textsubscript{2}
(criterion iv)

ClO\textsubscript{2} is a strong, but a rather selective oxidizer. Unlike other
oxidants it does not react (or reacts extremely slowly) with most
organic compounds of a living tissue. ClO\textsubscript{2} reacts rather fast,
however, with cysteine [22] and methionine [34] (two sulphur
containing amino acids), with tyrosine [23] and tryptophan [24]
(two aromatic amino acids) and with two inorganic ions: Fe$^{2+}$ and Mn$^{2+}$. It is generally assumed that the antimicrobial effect of ClO$_2$ is due mostly to its reactions with the previously mentioned four amino acids and their residues in proteins and peptides. In the peptide group it is important to mention glutathione – a small tripeptide containing cysteine – which is a major antioxidant in cells, with an intracellular concentration of 0.1-10 mM [35].

Margerum’s group [22–24] reported the following second order rate constants at pH 7 and 25 °C: cysteine 1×10$^7$ M$^{-1}$s$^{-1}$ >> tyrosine 1.8×10$^7$ M$^{-1}$s$^{-1}$ > tryptophan 3.4×10$^6$ M$^{-1}$s$^{-1}$. As can be seen, cysteine is the far most reactive amino acid because of its thiol group. As the above mentioned four amino acids and especially cysteine and biological thiols play a crucial role in all living systems, including microbes, it is impossible for any microbe to develop a resistance against chlorine dioxide.

As an important analogy we can mention that bacteria have never been able to become resistant against hypochlorous acid (HOCl) either, which is an important natural antiseptic used by neutrophils for millions of years. Neutrophils, a type of white blood cells, are phagocytes which kill the engulfed microbes by releasing numerous other substrates. Thus killing bacteria with HOCl is due to its reaction with sulfhydryl groups [18]. It is a logical assumption that ClO$_2$ is also a more drastic reagent and causes irreversible damage. For example ClO$_2$ oxidizes glutathione (GSH) mainly to glutathione disulfide (GSSG) [22] which can be reduced back to GSH easily in a natural way in the body. On the other hand, HOCl can attack disulfide bonds and oxidizes GSH mostly to glutathione sulfonamide (GSA) [44] causing an irreversible loss of the cellular GSH.

### Comparison of ClO$_2$ and HOCl as possible antiseptic agents

HOCl, like ClO$_2$, reacts rapidly with the sulphur containing amino acid residues of methionine and cysteine, the second order rate constant (at pH 7.4 and 22 °C) being 3.8×10$^7$ M$^{-1}$s$^{-1}$ and 3.0×10$^7$ M$^{-1}$s$^{-1}$, respectively, and also reacts with tryptophan (1.1×10$^6$ M$^{-1}$s$^{-1}$) and tyrosine (44 M$^{-1}$s$^{-1}$) [40]. However, unlike ClO$_2$, HOCl reacts rapidly with many other amino acid residues and even with peptide bonds [40], and many other compounds such as carbohydrates, lipids, nucleobases, and amines [41].

As we can see the important similarity is the fast reaction of both HOCl and ClO$_2$ with the SH group of cysteine. This is important because it is assumed that abolition of ATP synthesis and killing bacteria by HOCl is due to its reaction with sulfhydryl groups [18]. It is a logical assumption that ClO$_2$ can also stop the ATP synthesis as it reacts with the very same SH groups like HOCl.

At the same time, however, there are important dissimilarities between HOCl and ClO$_2$:

i) HOCl is much less specific and reacts rapidly with numerous other substrates. Thus killing bacteria with HOCl requires more reagent than with ClO$_2$.

ii) While ClO$_2$ evaporates rapidly from its aqueous solution and can reach and kill bacteria even through a gas phase, e.g. through an air bubble blocking a dental root canal [42], evaporation of HOCl is not significant. Thus HOCl stays at the disinfected area for a long time even after killing all bacteria which can cause inflammation there [43].

iii) HOCl is a more drastic reagent and causes irreversible damage. For example ClO$_2$ oxidizes glutathione (GSH) mainly to glutathione disulfide (GSSG) [22] which can be reduced back to GSH easily in a natural way in the body. On the other hand, HOCl can attack disulfide bonds and oxidizes GSH mostly to glutathione sulfonamide (GSA) [44] causing an irreversible loss of the cellular GSH.

### Sulphhydryl groups and circulation can protect multicellular organisms from ClO$_2$ inflicted irreversible damage

As it was mentioned, the ClO$_2$--SH group reaction has the highest rate constant among the ClO$_2$ – amino acid reactions. (Cysteine or GSH [22] reacts about 50 times faster than the runner up tyrosine.) Consequently, as long as some SH groups are present (mostly in the form of GSH), these groups react with ClO$_2$ rapidly protecting other amino acid residues from oxidative damage. Moreover the oxidation of SH groups to disulfide bonds can be reversed. An interesting example was presented by Müller and Kramer [45,46]. They found that the cytotoxic effect of povidone-iodine after a 30 min contact with murine fibroblast was only temporal: after a 24 hour culture without the antiseptic an unexpected revitalization of the fibroblasts was observed [45]. According to Winterbourn and co-workers [47], HOI (the reactive hydrolysis product of iodine) also oxidises GSH to GSSG but not to GSA. That parallelism between the reversible HOI-GSH and the ClO$_2$-GSH reactions raises the question whether an analogous revitalization might also be possible in the case of ClO$_2$. This question is all the more justified since in some animal experiments [16] rats were drinking water containing 200 ppm ClO$_2$ for 90 days but without developing any gastrointestinal problems. In those experiments all ClO$_2$ must have reacted with the animal tissues as it cannot evaporate from the stomach of the rats. To interpret that result it is reasonable to assume that SH groups transported by the circulation system of the rodent protected the epithelial cells in its gastrointestinal tract from an irreversible oxidation by ClO$_2$.

Above a certain limit, however, when a too high percentage of the protective SH groups is already oxidized, ClO$_2$ would inflict irreversible changes to the higher order protein structures by oxidizing the tyrosine and tryptophan residues [48]. That would certainly happen with the bacteria on the surface of an infected tissue as their GSH supply [26] can be rapidly exhausted by ClO$_2$. Mammalian cells below the surface, however, might survive being supported by the circulation which transports protective sulphydryl and other reductive compounds to the cells, continuously repairing or even revitalizing them.

Thus beside their size there is another important difference between single cell and more complex multicellular organisms: it is the circulation which can help the cells of a multicellular organism to survive while that type of help is not available for a bacterium.

### Conclusion

Chlorine dioxide is a size selective antimicrobial agent which can kill micron sized organisms rapidly but cannot make real
harm to much larger organisms like animals or humans as it is not able to penetrate deeply into their living tissues. Moreover the circulation of multicellular organisms can provide an additional protection to these organisms against ClO₂.

It is an aim of the present work to initiate clinical studies hoping that ClO₂ could be applied to treat various local infections, especially where bacterial resistance is a problem. We have already obtained an official permission [49] to start such studies.

Supporting Information

Information S1. This file contains the description of a reaction-diffusion (RD) model for the transport of ClO₂ in a medium containing reactive proteins, and its quasi steady state solution when the ClO₂ – substrate reaction is fast and when it is slow. Figure S1. Schematic ClO₂ and substrate concentration profiles in a hydrogel slab. Table S1. Data depicted in Figure 2 (the cumulative volume V of the 0.01 M Na₂S₂O₃ titrant added until time t). Table S2. Data depicted in Figure 3 (the cumulative volume V of the 0.01 M Na₂S₂O₃ titrant added until time t).

References


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