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DOI
10.1002/cctc.202001225

Publication date
2020

Document Version
Final published version

Published in
ChemCatChem

Citation (APA)
Yayci, A., Dirks, T., Kogelheide, F., Alcalde, M., Hollmann, F., Awakowicz, P., & Bandow, J. E. (2020). Microscale Atmospheric Pressure Plasma Jet as a Source for Plasma-Driven Biocatalysis. ChemCatChem, 12(23), 5893-5897. https://doi.org/10.1002/cctc.202001225

Important note
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Microscale Atmospheric Pressure Plasma Jet as a Source for Plasma-Driven Biocatalysis

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The use of a microscale atmospheric pressure plasma jet (μAPPJ) was investigated for its potential to supply hydrogen peroxide in biocatalysis. Compared to a previously employed dielectric barrier discharge (DBD), the μAPPJ offered significantly higher H₂O₂ production rates and better handling of larger reaction volumes. The performance of the μAPPJ was evaluated with recombinant unspecific peroxygenase from Agrocybe aegerita (raaeUPO). Using plasma-treated buffer, no side reactions with other plasma-generated species were detected. For long-term treatment, rraaeUPO was immobilized, transferred to a rotating bed reactor, and reactions performed using the μAPPJ. The enzyme had a turnover of 36,415 mol mol⁻¹⁻¹ and retained almost full activity even after prolonged plasma treatment. Overall, the μAPPJ presents a promising plasma source for plasma-driven biocatalysis.

Main text

Peroxygenases perform a range of hydrogen peroxide (H₂O₂)-dependent oxyfunctionalization reactions such as enantioselective hydroxylations and epoxidations.[1] Their high selectivity and operational stability makes peroxygenases appealing enzymes for biocatalysis. However, like all heme-containing enzymes, peroxygenases suffer from inactivation by H₂O₂.[2] Several approaches exist to generate H₂O₂ gradually in situ, thereby lowering the working concentration of H₂O₂ and mitigating the issue of inactivation as well as dilution of the reaction solution.[3]

One approach makes use of atmospheric pressure plasmas.[4] In general, plasmas are generated by high electric fields, accelerating free electrons that collide with atoms or molecules in the gas phase, which leads to the formation of metastable as well as excited species and ions.[5] The short-living reactive species react with each other or with surrounding gas atoms or molecules to eventually yield less reactive species.[6] One of the species produced in plasma-treated aqueous liquids is H₂O₂ that can then be used to drive peroxygenase-based biocatalysis.

We previously showed that the H₂O₂ in plasma-treated buffers can drive biocatalysis with the in vitro-evolved, recombinant UPO from Agrocybe aegerita (raaeUPO).[4] When enzyme and substrate were treated together with plasma, however, the yield of product was significantly reduced as compared to a catalysis scheme in which plasma treatment and subsequent biocatalysis were uncoupled. Since the enzyme was quickly inactivated by plasma treatment with a dielectric barrier discharge (DBD), immobilization was used to prolong the lifetime of the enzyme. However, immobilization did not significantly improve product formation under direct treatment. Since only small volumes (μL scale) could be treated, mixing of the reaction solution during plasma treatment was not feasible, thereby limiting substrate supply to the enzyme and presumably turnover.

In this study, we addressed the aforementioned shortcomings by going into the micro-scale. In a first experiment, the influence of the surrounding gas on the H₂O₂ production rate of a DBD was investigated. An in-house built DBD,[7] employing comparable electrode geometry and plasma parameters as the previously used Cinogy PlasmaDerm source,[8] was placed in different atmospheres and used to treat 110 μL of phosphate buffer. Immediately after treatment, the H₂O₂ concentration was measured with a colorimetric assay. Using an argon atmosphere to ignite the plasma gave the highest H₂O₂ concentration as compared to synthetic air or nitrogen (Figure 1). In argon atmosphere, after 5 min 13.7 mM of H₂O₂ was measured whereas only 3 mM and 4.7 mM H₂O₂ were observed under synthetic air and nitrogen, respectively, under otherwise identical conditions (Figure 1). It is worth mentioning that the H₂O₂ accumulation was linear during the 5 min treatment in all cases.
Since argon is an atomic gas, the collision of accelerated electrons and gas particles may not lead to molecular vibration or rotation and therefore increases the formation of metastables and ions, which in turn increases H$_2$O$_2$ production.

Next, we tested whether the high production rate of H$_2$O$_2$ under argon atmosphere persists when treating larger volumes of liquid. Using the same setup, 5 mL of phosphate buffer were treated and analyzed. The absolute production rate of H$_2$O$_2$, i.e. the total amount of H$_2$O$_2$ molecules produced per minute, for 5 mL was significantly lower than for 110 μL, indicating that the surface-to-volume ratio influences H$_2$O$_2$ generation (Figure 2). Nevertheless, the DBD setup used here did not allow for a wider vessel to be treated in a pure argon atmosphere so that no further experiments were conducted to investigate the scalability of biocatalysis driven by this source.

Since the DBD geometry posed some restrictions on the sample volume and distance to the source as well as H$_2$O$_2$ production rate, a different kind of plasma source was tested, namely a microscale atmospheric pressure plasma jet (µAPPJ). A comparison of both plasma geometries is shown in Figure 3. In the µAPPJ, the plasma is ignited between two electrodes with a vertical noble gas flow (in this case He) that disperses the created reactive species. While in a DBD setup the sample is in direct contact with the plasma, with the µAPPJ the sample is only exposed to the plasma effluent, i.e. the reactive species transported by the gas flow. Since the plasma is remote from the treated sample, the µAPPJ enables treatment over a broad range of sample volumes and formats.

Previous studies with the µAPPJ showed that by employing water vapor in the feed gas the generation of H$_2$O$_2$ is greatly enhanced. Therefore, we first measured the H$_2$O$_2$ production rate and whether it improved with increased water vapor content. A part of the feed gas was passed through a bubbler containing deionized water and mixed with the remaining dry feed gas before going through the electrode. When 10% of the total feed gas flow were passed through the bubbler, the H$_2$O$_2$ production rate already increased significantly (Figure 4).

The absolute H$_2$O$_2$ production of the µAPPJ with 25% relative humidity was comparable to that of the DBD in argon and approx. 15× higher than that of the DBD in ambient air (see Figure 2 and Table S1 for details). When more than 25% of the gas feed were routed through the bubbler, the plasma did not ignite anymore when applying the standard voltage of 230 V. Therefore we used 25% of water-treated He for all subsequent experiments. H$_2$O$_2$ concentration also increased linearly with the treatment time, showing that the production rate stays constant (Figure S1). Adding minor amounts of oxygen to the feed gas had no significant effect on H$_2$O$_2$ production (Figure S2).

In a biocatalysis setting, it would be beneficial to tailor the plasma and thereby the supplied amount of H$_2$O$_2$ to match the needs and limitations of the enzyme employed. We therefore...
tested whether $\text{H}_2\text{O}_2$ production can be tuned by modifying the voltage. Generation of $\text{H}_2\text{O}_2$ was found to correlate linearly with the applied voltage, which corresponds to input power (Figure 5). The ability to quickly tune $\text{H}_2\text{O}_2$ production rates is a decisive advantage over some other in situ methods, e.g. enzymatic $\text{H}_2\text{O}_2$ generation systems.

After having established that the $\mu$APPJ produces suitable amounts of $\text{H}_2\text{O}_2\mu$, we first checked whether plasma-treated buffer was a reasonable resource to drive biocatalysis. The investigated reaction was the well-known hydroxylation of ethylbenzene (ETBE) to (R)-1-phenylethanol ((R)-1-Phol) by rAaeUPO. This enzyme showed remarkable turnover numbers (TONs) and enantioselectivity in previous studies and was used in conjunction with plasma before. After treating 5 mL of buffer with the $\mu$APPJ for 15 min, ETBE and rAaeUPO were added and allowed to react for 15 min. Both $\text{H}_2\text{O}_2$ produced with the $\mu$APPJ and a diluted $\text{H}_2\text{O}_2$ stock solution with the same concentration as in plasma-treated buffer yielded approx. 1.1 mM product (92% ee) after enzymatic conversion, showing that other possible side products of plasma treatment, like peroxynitrite, nitrite, or nitrate [9], did not have a negative effect on the reaction (Figure 6). No overoxidation of 1-Phol to acetophenone was observed in GC chromatograms. Also, no background activity was observed for plasma-treated buffer without the enzyme.

In order to couple plasma treatment and catalysis while retaining enzyme activity, rAaeUPO was then immobilized by covalent binding to support beads. The beads were transferred to a small-scale rotating bed reactor that provides mixing through rotation, allowing for high substrate flow through the bead layer. The rotating bed reactor was placed into a suitable vessel filled with 5 mL of buffer containing 50 mM of ETBE and treated with the $\mu$APPJ. Aliquots were withdrawn and analyzed by GC, revealing that after an initial phase with high conversion, the turnover rate declines at around 3 mM of (R)-1-Phol (Figure 7). In this setup, a TON of 23,037 mol$_{(\text{R})\text{-1-Phol}}$ mol$^{-1}$$\text{rAaeUPO}$ was achieved.

Generally, turnover stalling can be explained by three major causes: reduced enzyme activity, substrate depletion, or product inhibition. After 80 min of treatment, the immobilized enzyme was extracted and checked for activity ex situ. Only a negligible loss of activity was found, showing that enzyme activity was not the reason for decreased turnover (Figure S3). Next, the performance of the enzyme was tested in the same rotating bed reactor system when 4 mM of racemic 1-Phol,
approximating the extrapolated final concentration shown in Figure 7, were added from a stock solution. Enzyme activity decreased to ~61% (Table S2), indicating that high concentrations of the product 1-Phol indeed affected turnover. However, the increase in product concentration from 70 to 80 min of treatment in Figure 7 corresponds to only ~30% of the initial activity, showing that product inhibition is not the only cause for decreased turnover.

The μAPPJ is operated with 1.4 slm gas flow. Because the effluent hits the surface of the treated solution, it seemed likely that substrate evaporation limits catalysis. While the overall buffer volume stayed constant for long periods of time (~10% loss after 30 min), the volatile substrate ETBE appeared to evaporate. Since ETBE is hydrophobic and only dissolves into the liquid at quite low concentrations, the majority of the substrate floats on top of the reaction solution in droplets, even when the reaction mixture is stirred. These droplets were observed to quickly evaporate due to the high gas flow to the surface which could negatively affect the outcome of the experiment presented in Figure 7 in which the substrate is not added at high concentrations at the beginning of the reaction and may be depleted at prolonged treatment times.

Consequently, direct biocatalysis using the rotating bed reactor was repeated while replacing the entire reaction solution after every cycle of 10 min (Figure 8).

The obtained (R)-1-Phol concentration stayed constant over 8 cycles (total of 80 min accumulated treatment time), indicating that both substrate depletion as well as product inhibition were alleviated. The TON for this system was 36,415 mol_{(R)-1-Phol} mol^{-1}_{μAPPJ}, which is comparable to previously published results. Here, however, enzyme activity was not exhausted so that much higher TONs are to be expected.

In summary, we showed that a μAPPJ is a suitable plasma source for plasma-driven biocatalysis. Compared to the DBD, the μAPPJ is advantageous because larger volumes can be effectively treated and biocatalysis with direct treatment using the plasma effluent is now feasible. However, the μAPPJ used here requires the use of expensive feed gases, such as helium or argon, which considerably increases the cost of running the system. Jet-based plasma sources that operate in ambient air have successfully been designed and would be beneficial for biocatalysis, combining cost-effective operation and favorable source geometry.

At this point, the solubility of hydrophobic substrates needs to be addressed as well to make this system truly valuable for preparative scale.

Experimental section

Plasma sources

The DBD was used essentially as described before at 24 kV_{pp} and 300 Hz. A detailed account of the DBD can be found elsewhere. To provide different gas atmospheres, a lateral gas flow was applied at 2 slm. When 5 mL were treated, a stainless steel wire loop was placed inside the glass vessel and connected to the ground.

The μAPPJ was operated at 230 V_{pp} and 13.56 MHz with a combined flow of 1.4 slm He. The gas feed was split and partially routed through a bubbler containing deionized water at room temperature. Both lines were merged in a T-piece before entering the electrode chamber.

H₂O₂ measurements

Immediately after treatment, samples were withdrawn and diluted to an appropriate concentration with deionized water. To 200 µL of the diluted sample, 12.5 µL of the reagents 1 and 2, supplied by a commercially available kit, were added and left to react for 5 min (Spectroquant H₂O₂, Merck, Germany). Absorption was measured at 455 nm and concentration determined using a calibration curve.
Enzyme preparation

In vitro-evolved, recombinant rAaeUPO was purified essentially as described before.[5] The supernatant of a Pichia pastoris expression culture was subjected to microfiltration and single-step ion exchange chromatography, yielding the purified protein.[6] Briefly, HA403 M beads (Resindion, Binasco, Italy) were washed twice with water and incubated with phosphate buffer (pH 7) containing 0.5% glutaraldehyde. After 1 h, beads were washed three times with buffer and enzyme was added at 0.5 nmol per 100 mg beads. Immobilization was allowed to proceed overnight at 8°C. Binding efficiency was checked by measuring enzyme activity in the supernatant and was >80% in all cases. Roughly 150 mg of protein-loaded beads were then transferred to a rotating bed reactor that was build in-house by 3D-printing (dimensions: ø2 cm × 0.7 cm). The reactor was designed with a snap-on lid to enable extraction of the enzyme and reuse of the reactor. The final concentration of rAaeUPO in 5 mL of buffer was approx. 125 nM (see Table S3 for details).

Concentration of ETBE

To generate plasma-treated buffer, 5 mL of buffer were treated with the μAPPJ for 15 min with constant stirring. Subsequently, 50 μM of ETBE were added and the reaction solution was mixed for 15 min by overhead rotation to allow for the substrate to go into solution. Then, 50 μM of rAaeUPO were added and the solution incubated at 30°C for 15 min with constant shaking.

When the rotating bed reactor was used, 5 mL of buffer containing 50 μM of ETBE were mixed and placed into a suitable vessel with the rotating bed reactor. Plasma exposure was conducted at approx. 4 mm distance between the nozzle and the liquid surface.

Analysis of (R)-1-Phol

Aliquots of 150 μL were withdrawn and mixed with the same volume of ethyl acetate containing 2 mM of 1-octanol as internal standard. The organic phase was transferred to a new vial, dried with MgSO₄, and subjected to gas chromatography. Samples were analyzed using a Shimadzu 2010 system with a Hydrodex C, 10 min). Concentrations were determined by a calibration curve with racemic 1-Phol.

Acknowledgements

We thank Marco Krewing for fruitful discussions and the workshop of the Department of Biology and Biotechnology (Bochum) for constructing the rotating bed reactor. Funding was provided by CRC1316-1 to JEB and PA and RTG2341 to JEB. Open access funding enabled and organized by Projekt DEAL.

Keywords: atmospheric pressure plasma · biocatalysis · hydrogen peroxide · peroxygenase · plasma chemistry

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Plasma jets and enzymes! A microscale atmospheric pressure plasma jet was employed to generate H$_2$O$_2$ for biocatalysis. Unspecific peroxigenase from *Agrocybe aegerita* was immobilized and used in a rotating bed reactor system. Conversion of ethylbenzene to (R)-1-Phol using plasma-generated H$_2$O$_2$ was performed with high enantioselectivity and satisfactory TON.