Investigation of cold plasma treatment on antimicrobial peptides as strategy for selective bacterial targeting

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Abstract: Cold atmospheric pressure plasma for the intentional modification of biomolecules such as peptides represents an area with significant application potential. This study focuses on the reactivation of antimicrobial peptides by targeted bond cleavage after exposure to cold plasma. First results showed that treatment with a dielectric barrier discharge source leads to the required bond cleavage as well as an unfavourable inactivation of the peptides in experiments with *Bacillus Subtilis*.

Keywords: DBD, antimicrobial peptides, plasma cleavage, plasma medicine

1. Introduction and Objective

The investigation and application of cold atmospheric pressure plasma (CAP) in the medical field for antimicrobial treatment[1] as for chronic wounds[2] or anti-cancer therapy[3] is gaining increasing attention.
One form of plasma source in these areas is the dielectric barrier discharge (DBD).[4][5] Operation under ambient air conditions allows the generation of various reactive oxygen and nitrogen species (RONS) with the DBD.[6][7] These reactive species are not only of interest for the treatment of bacterial diseases but also for chemical modification. This opens up potential to use cold plasma treatments for targeted modification of small biomolecules. Initial investigations have already shown that the treatment of glutathione (GSH) and glutathione disulphide (GSSG) with DBD leads to modifications on the sulfur moiety.[8] In case of GSSG, a cleavage of the disulphur bridge was also identified under plasma conditions.[9] The question therefore arises as to what potential these properties may hold in the field of biomolecules. Small biomolecules such as antimicrobial peptides (AMP) are interesting candidates for combination with cold plasma. The group of arginine (R) and tryptophan (W) -rich (RW)n peptides, which have already demonstrated antimicrobial properties and low cytotoxicity[10] in studies, also belong to this category of AMPs.[11][12] Even though the mode of action is not yet fully explained, it has been shown that the positive residue of arginine interacts with the negatively charged membrane of the bacteria by electrostatic attraction and thereby causes an increased permeability of the membrane. The behavior is different with the tryptophan residue, which can interact with the lipid bilayer through hydrophobic interactions. In combination, these two effects result in the destabilization of the cell membrane and pore formation, which in turn leads to bacterial cell death.[13][14][15]
Therefore, we designed a peptide containing $(RW)_3$ which is prevented from interacting with the bacterial cell by a coupled antagonist with opposite charge. The 3,3-dithio-dipropionic acid linker used to bridge the two sequences also represents the breaking point, which should lead to the release of the active $(RW)_3$ peptide through treatment with cold atmospheric plasma. (Fig.1)

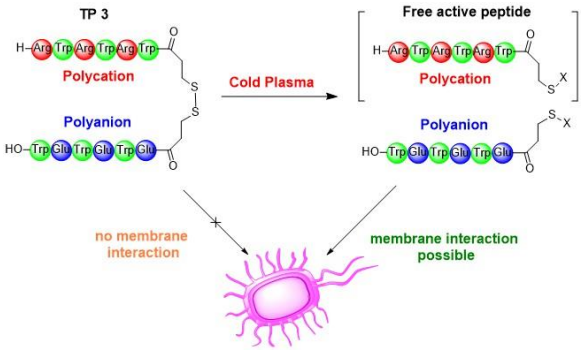

Fig. 1: Concept for the activation of antimicrobial peptides by using cold plasma. The $(RW)_3$ unit is bridged to the antagonistic $(EW)_3$ unit (E = glutamic acid) by a labile disulfur linker to form **TP 3**. By the selective cleavage of the disulfur bridge, the active $(RW)_3$ peptide should be released.

The $(RW)_3$ sequence (**TP1**) represents the antimicrobial active peptide, whereas the $(EW)_3$ peptide (**TP2**) should have no effect on the bacterial growth. The two combined peptides (**TP3**) represent the main target, which ideally should have no effect on the growth of the bacteria before treatment with cold plasma.

2. Methodology

The target peptides **TP 1 - TP 3**, presented in Fig.2, were synthesized by solid phase peptide synthesis according to the Fmoc strategy.[16] Wang resin was used as solid support for each target peptide. The synthesized peptides were purified by high pressure liquid chromatography (HPLC) and characterized by electrospray ionization mass spectrometry (ESI-MS).

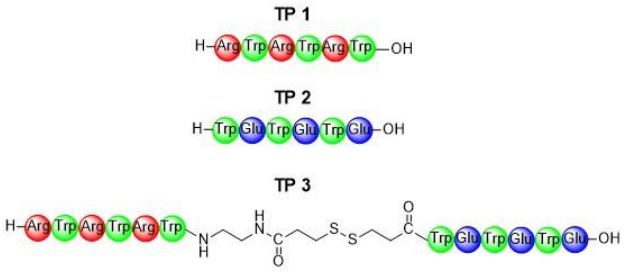

Fig. 2: List of synthesized target peptides **TP 1 – TP 3** for bacteria and plasma studies.

For the cold atmospheric plasma experiments, a DBD[17] plasma source was used. The treatment was carried out under ambient air conditions and treatment times of 10 - 120 s were investigated. First, the 3,3-dithio-dipropionic acid linker which is used for **TP 3** was tested for its stability under plasma conditions. For this purpose, we used a Si-wafer to apply the substances dissolved in aqueous medium. The linker was then analyzed for modifications using HPLC and ESI-MS. The plasma-treated and untreated samples were then used for growth experiments on *Bacillus subtilis* using a plate reader. Therefore, the optical density (OD) is measured to obtain information about the growth behavior of the bacteria under different conditions as a dependency of time.[18] In the first experiments, the inhibitory effect of the peptides **TP 1** and **TP 3** was investigated in dependence on their concentration. In the further course, the influence of the DBD-treated peptides on the growth of *Bacillus subtilis* was investigated under the same conditions.

3. Results and discussion

The synthesis of peptides **TP 1 - TP 3** were confirmed by ESI-MS analysis, with purity verified by HPLC. The experiments on the influence of the peptides on the growth rate of *Bacillus subtilis* showed no influence after adding the antagonistic peptide **TP 2**. The antimicrobial active peptide **TP 1** showed a temporal shift in growth, depending on the concentration used, up to the complete suppression of growth of the bacteria. The tests on the main target **TP 3** showed less influence on growth compared to peptide **TP 1**. With the data obtained, we investigated the difference in the influence of the peptides on growth after treatment with CAP in relation to the bacteria. The peptides **TP 1 - TP 3** showed no influence on the growth of the bacteria after plasma treatment (Fig.3). The influence of the peptide **TP 1** on the growth of the bacteria could be shown in variation of the concentration as expected, whereas **TP 2** shows no differences to the control group. However, it is interesting to look at the **TP 3** peptide before plasma treatment, as a delay in growth can be observed, which is, weaker than the inhibitory effect of compound **TP 1**.

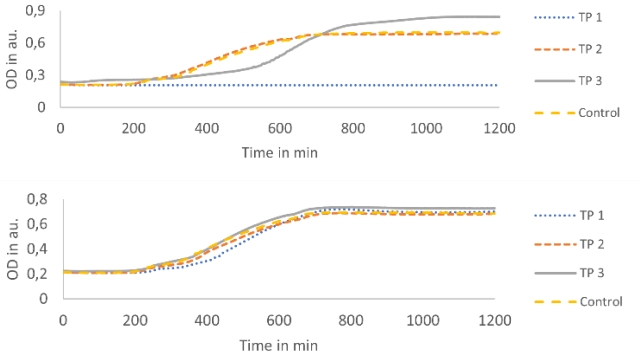

Fig. 3: Growth curves of *B. subtilis* with addition of **TP 1-TP 3**. The peptides were added at t = 0, whereby a concentration of 46.0 µmol/l was selected as the standard for **TP1-TP3**. Top Graph: Growth curve of *B. Subtilis* after addition of peptides without previous DBD treatment. Bottom Graph: Growth curve of *B. subtilis* after addition of peptides with previous DBD treatment.

This can be explained by a possible cleavage of the disulfur bridge in the biological medium. This process could release part of the active sequence and promote inhibition of bacterial cell growth. However, minor modifications to the linker could overcome this obstacle. The lack of effect of the peptides **TP 1** and **TP 3** on growth after plasma treatment suggests a structural change in the peptides through chemical reactions, which supposedly lose their ability to attach to the cell membrane. Since both the amino acid tryptophan and arginine have an impact on cell attachment and followed cell death, the plasma-induced change of **TP 1 -TP 3** needs to be analyzed and identified to gain a better understanding of peptide stability under cold atmospheric plasma conditions.

4. Conclusion

The results of the investigations on the influence of the peptides on the growth of *Bacillus subtilis* showed that **TP 1 – TP 3** were no longer active after plasma treatment. On the other hand, an inhibitory effect could be shown for **TP 1** as well as for **TP 3** without plasma treatment. This inactivation of the peptides suggests that there is only limited stability to the plasma treatment, which thus not only causes the selective cleavage of the disulfur bond, but also a structural change, for example in the side chain of the amino acids. The future focus of the investigation should therefore be on the treated peptide as well as the individual amino acids to detect structural changes that lead to inactivation. Switching the experiments to another plasma source with the possibility of milder treatment conditions, using different gases, could be another option for controlled and selective chemical modifications to the model peptides.

5. Acknowledgements

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