Designing cold plasma cleavable RGD peptide bioconjugates for targeted tumor therapy

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Abstract: In this work, two RGD (Arg-Gly-Asp) peptides linear RGDfK and cyclic c(RGDfK) were synthesized and conjugated to the metal complex ferrocenecarboxylic acid through amide bond formation, resulting in a monomeric bioconjugation. Both bioconjugates contain a disulfide bridge between peptide and metal complex. The time- dependent chemical modifications of the linear and cyclic peptide bioconjugates with an implemented disulfide bridge by cold plasma treatment were investigated. ESI-MS spectra of both bioconjugates verify cleavage of the disulfide bridge after 1 min of cold plasma treatment, while 4 min cold plasma treatment reveal difficult-to-handle chemical modifications which could not be assigned.

Keywords: bioconjugates, cell-targeting peptides, solid-phase peptide synthesis (SPPS), dielectric barrier discharge (DBD)

1. Introduction

Cancer is a multifactorial disease that results from a myriad of genetic and environmental factors. Platinum anticancer drugs are widely used for chemotherapy of various cancer diseases.[1] However, those anticancer agents constitute several significant limitations, such as poor selectivity and growing tumor resistance. Therefore, enhancing the tumor selectivity has become a major goal for the development of novel cytotoxic agents for tumor therapy. Hence, a new research field with focus on targeting and drug delivery systems (DDS) for metallodrugs were developed.[2,3] Specifically the functionalization of metallodrugs is aimed at improving tumor selectivity by minimizing systemic toxicity and enhancing their cellular accumulation to overcome tumor resistance. [4]

Peptides can be also utilized to target specific receptors, so called cell targeting peptides (CTP).[5] RGD peptides with an amino acid sequence of Arg-Gly-Asp are widely known for their selectivity and affinity towards $\alpha v\beta_3$ integrin receptor and therefore, highly favored in targeted cancer therapy. [6,7] The $\alpha v\beta_3$ integrin is critically involved in the processes of angiogenesis, metastasis and invasion of solid tumors and thus overexpressed in tumor cells. A few of these RGD peptide derivatives show impressive results in preclinical trials and RGD targeted radiotracers have been already successfully tested in humans for visualization of $\alpha v\beta_3$ integrin, which demonstrates the feasibility of this approach.[8]

In past years the effectivity of drug delivery systems was enhanced by the addition of physical energy to conventional DDSs.[9,10] These techniques have the potential to be utilized in different biological and medical applications as an advanced targeted drug delivery system (TDDS), decreasing the current limitations and undesired side effects.

Atmospheric cold pressure plasmas (ACPP) are gaining increasing interest in biomedical trials for enhanced wound healing[11] and skin disinfection[12]. A typical plasma source for wound healing is a dielectric barrier discharge (DBD), which allows direct contact between skin and active plasma zone shown in figure 1.[13] DBDs are mostly generated directly in ambient air, relinquishing the need of a special gas system.[14,15] The DBD generates under ambient conditions reactive oxygen and nitrogen species (RONS), which further interact with the targeted area. In our previous studies, we have demonstrated the plasma-induced chemical modifications of cysteine.[16]. Furthermore, we identified the chemical effects of DBD on glutathione (GSH) and glutathione disulphide (GSSG) as the most important redox pair in organisms responsible for detoxification of intracellular reactive species.[17] Studies revealed that all three possible species of S-oxidized GSH (GSOH, GSO_2H, GSO_3H) are produced with GSO_3H being the most prominent signal after 5 min of treatment. Moreover, we investigated the time-dependent chemical modifications of GSH and GSSG in the presence of iron(II) and iron(III) complexes caused by DBD under ambient conditions.[18] For both compounds GSH and GSSG, GSO_3H was observed as the only oxidation species.

In connection with our previous studies, we focus on the development and synthesis of novel RGD peptide bioconjugates and their activation through cold atmospheric pressure plasma treatment. Therefore, a linker containing a disulfide bridge will be inserted between peptide and metal complex which is potentially activated through S-S bond cleavage during plasma treatment (Figure 1). The peptide RGDfK will be synthesized in linear and cyclic version to compare stability and activity under cold plasma conditions. The incorporation of lysine will provide the platform for the coupling of plasma-cleavable linkers. Ferrocenecarboxylic acid will be the metal complex of interest for bioconjugation. Cold plasma treatment with several treatment times will be applied to investigate optimal cleavage conditions of the disulfide

bridge. The linear peptides, cyclic peptides and bioconjugates as well as the plasma treated samples will be analyzed via ESI-MS and analytical HPLC.

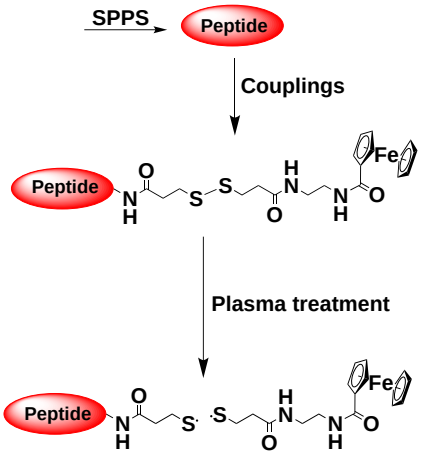

Fig. 2. Overview of the planned synthesis route: starting with solid phase peptide synthesis, coupling of both linkers including a disulfide bridge, bioconjugation with ferrocenecarboxylic acid and cold plasma treatment afterwards.

2. Methodolgy

All chemicals used were purchased from Sigma-Aldrich, Fisher Scientific GmbH, IRIS Biotech GmbH, Alfa Aesar and were utilized without further purification.

High-performance liquid chromatography (HPLC) was performed on Knauer Smartline system, using a Reprosil-Pure C_{18} reverse-phase column (250x4.6mm). Electron spray ionization mass spectra (ESI-MS) were obtained on Bruker Esquire 6000 mass spectrometer.

The plasma experiments in this study were carried out with a dielectric barrier discharge, which consists of a copper electrode covered with aluminium oxide (Al_2O_3). The electrode has a diameter of 10 mm and the distance between the driven electrode and the sample was kept constant at 1 mm. The samples were placed on a grounded aluminium plate and ambient air was used as process gas. The experimental setup is described in more detail in Kogelheide et al.. [16] The electrode was driven with a pulsed power supply. For the experiments in this study the repetition frequency was set to 300 Hz and the amplitude of the HV pulse to 24 kVpp.

3. Results

For the cold plasma experiments, the linear peptide bioconjugate was synthesized via standard Fmoc solid phase peptide synthesis. An Overview of the synthesis route is shown in Figure 3. The HPLC retention times and ESI-MS data verifying the synthesis steps for the linear peptide are shown in Table 1.

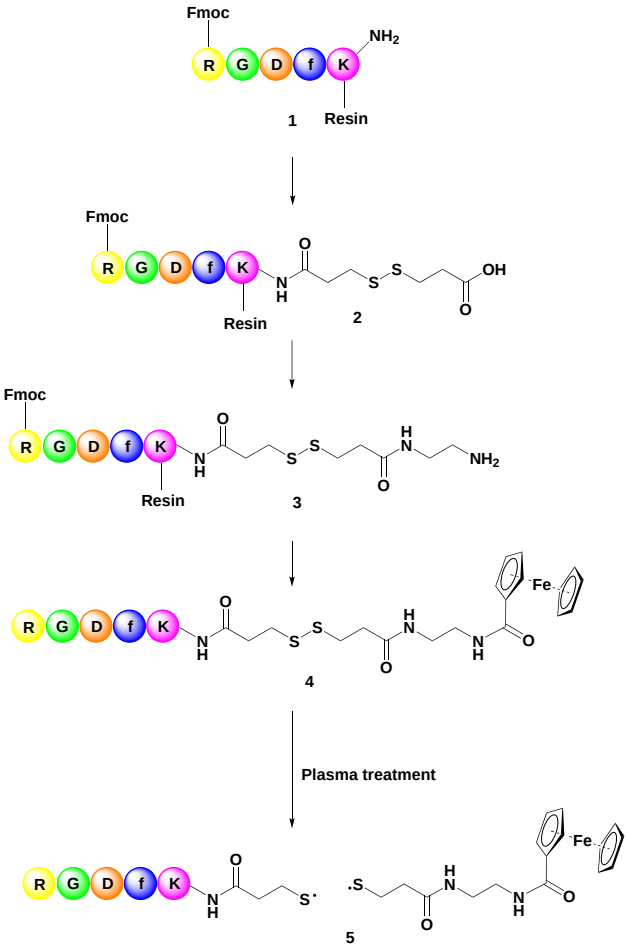

Fig. 3. Synthesis route for the linear RGD peptide bioconjugate

Table 1. HPLC retention time and ESI-MS data of the linear peptide couplings.

Compounds	Retention time (T_R)	*m/z* obsv.	*m/z* calcd.
RGDfK	5.8 min	622.01	621.69
RGDfK-SS-OH	6.5 min	814.51	814.32
RGDfK-SS-NNH_2	6.3 min	857.47	856.25
RGDfK-SS-NN-$FeCp_2$	7.7 min	1068.52	1068.57

To examine the stability of the peptides and their modifications, cold plasma experiments were performed with DBD. For this purpose, the samples were dissolved in HPLC grade water, placed on cleaned silicon wafers and treated with a DBD for 1 min, 2 min, 3 min, 4 min and 5 min. After treatment, the samples were analyzed via ESI-MS. To find out whether the pure linear peptide is stable under plasma conditions, the impact of cold plasma treatment on peptide **1** which is shown in figure 3 was investigated after different treatment times. The ESI-MS spectra of the samples treated for 1 min, 2 min, 3 min, 4 min and 5 min, all showed signals at m/z = 311.85, corresponding to the $[M+H]^{2+}$ ion peak of the RGDfK

peptide **1** and a signal m/z = 622.23 which can be assigned to the $[M+H]^+$ peak of peptide **1**. This clearly demonstrates the stability of the peptide towards cold plasma over longer treatment times. Moreover, the impact of cold plasma treatment on the linear peptide **2** containing 3,3-dithiodipropionic acid was investigated after different treatment times. The ESI-MS spectrum of peptide **2** after 1 min showed a signal at m/z = 354.85 which corresponds to $[M+H]^{2+}$ ion peak of the RGDfK-SH moiety, indicating a successful cleavage of the disulfide bridge. After 2 min of plasma treatment, two signals could be observed. The first signal at m/z = 354.95 corresponds to the $[M+H]^{2+}$ ion peak of RGDfK-SH moiety and the second signal at m/z = 759.04 can be assigned to the $[M+H]^+$ peak of RGDfK-SO_3H, showing another oxidation product of the plasma cleaved disulfide bridge. The ESI-MS spectrum after 3 min revealed only a signal at m/z = 759.09 corresponding to the $[M+H]^+$ ion peak of the RGDfK-SO_3H oxidation state. ESI-MS spectra after 4 min and 5 min of plasma treatment did not show any signal which could be assigned to expected modifications. This might be due to long treatment times causing difficult-to-handle modifications, which cannot be definitely assigned. Results of the plasma treated linear peptide containing 3,3-dithiodipropionic acid are summarized in Table 2 and shown in Figure 4.

Table 2. ESI-MS data of the cold plasma treated linear peptide containing 3,3-dithiodipropionic acid

Treatment time	Ion peak	*m/z* obsv.	*m/z* calcd.
1 min	$[M+H]^{2+}$	354.85	354.53
2 min	$[M+H]^+$ $[M+H]^{2+}$	354.95 759.04	354.53 758.01
3 min	$[M+H]^+$	759.09	758.01

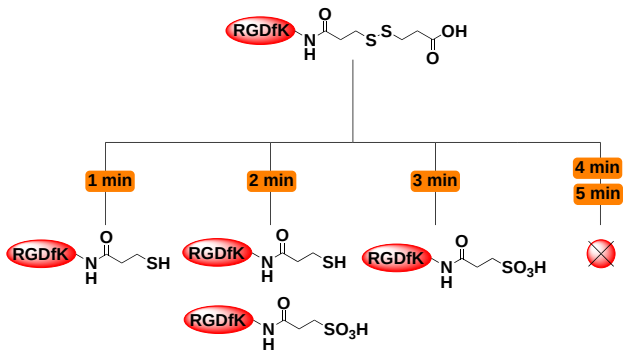

Fig. 4. Chemical modifications of the linear peptide shown in Figure 3 with 3,3-dithiodipropionic acid coupled at lysine after 1 min, 2 min, 3 min, 4 min and 5 min of cold plasma treatment.

Lastly, cold plasma experiments on peptide **4** were performed for different treatment times. ESI-MS spectra after 1 min, 2 min, 3 min, 4 min and 5 min did not show any remarkable signals which could be assigned to expected modifications. These results suggest on the one hand, the generation of difficult-to-handle species after plasma treatment and on the other hand, non- optimal reaction conditions for the linear peptide bioconjugate.

Results of the cold plasma experiments on cyclic RGD peptide bioconjugate will be presented during the conference.

4. Conclusion

In this work, the successful synthesis of a novel linear peptide bioconjugate with implemented disulfide bridge was determined. Moreover, cleavage of the disulfide bridge between peptide and metal complex after 1 min of cold plasma treatment on the linear peptide bioconjugate was demonstrated.

Synthesis of the linear peptide bioconjugate was performed by synthesis of the linear pentapeptide and subsequent coupling of the linkers 3,3´-dithiodipropionic acid, ethylenediamine and the metal complex ferrocenecarboxylic acid. All steps were characterized and verified by ESI-MS and analytical HPLC evincing a successful synthesis of all conjugation steps. However, the solubility of the peptide conjugates decreases after each coupling in HPLC grade water which is used for cold plasma experiments and mass spectrum analysis. This leads to ESI-MS spectra with lower intensity as expected and precipitation building during plasma treatment, as water evaporates with increasing treatment times.

Plasma experiments of the linear peptide bioconjugate, revealed cleavage of the disulfide bridge after 1 min of plasma treatment showing a thiol species as cleavage product. After 2 min of plasma treatment an additional species could be detected, which was identified as sulfonic acid derivatives of the cleaved disulfide bridge, which is consistent with previously plasma treated biomolecules such as cysteine, GSH and GSSG, as all these biomolecules were oxidated through the influence of cold plasma. After 3 min the thiol species was further oxidized, which led to the fact that only the sulfonic acid species was observed as the only species. Plasma treatments of 4 min and 5 min show no signals which could be assigned, indicating difficult-to-handle modifications which cannot be allocated. Potential complications of the plasma treated samples after 4 min and 5 min could arise from the used electro-spray ionization mass spectrometer, as the detection sensitivity is too low, to detect complex species in smaller concentrations. For future experiments, the plasma treated sample analysis should be supplemented by a high-resolution mass spectrometer to detect more complex species.

In future, the solubility of the conjugates needs optimization to enhance the intensity of the mass spectra. Additionally, HPLC analysis should be performed with the plasma treated samples. Moreover, the cytotoxicity of both RGD peptide bioconjugates as well as the plasma treated

versions must be investigated. Furthermore, other metal complexes suitable for targeted tumor therapy should be investigated and cold plasma experiments must be performed. Suitable metal complexes for plasma conditions are Pt(IV) or Au(III) complexes, as the RONS could possibly oxidize other metal complexes, which possess higher oxidation states.

5. Acknowledgements

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6. References

[1] R. J. Browning, P. J. T. Reardon, M. Parhizkar, R. B. Pedley, M. Edirisinghe, J. C. Knowles, E. Stride, *ACS Nano* **2017**, *11*, 8560–8578.

[2] G. Tiwari, R. Tiwari, S. Bannerjee, L. Bhati, S. Pandey, P. Pandey, B. Sriwastawa, *Int J Pharm Investig* **2012**, *2*, 2.

[3] S. S. Dharap, Y. Wang, P. Chandna, J. J. Khandare, B. Qiu, S. Gunaseelan, P. J. Sinko, S. Stein, A. Farmanfarmaian, T. Minko, *Proc Natl Acad Sci U S A* **2005**, *102*, 12962–12967.

[4] H. Khan, A. Badshah, M. Said, G. Murtaza, J. Ahmad, B. J. Jean-Claude, M. Todorova, I. S. Butler, *Appl Organomet Chem* **2013**, *27*, 387–395.

[5] R. L. Juliano, R. Alam, V. Dixit, H. M. Kang, *WIREs Nanomedicine and Nanobiotechnology* **2009**, *1*, 324–335.

[6] M. Nieberler, U. Reuning, F. Reichart, J. Notni, H.-J. Wester, M. Schwaiger, M. Weinmüller, A. Räder, K. Steiger, H. Kessler, *Cancers (Basel)* **2017**, *9*, 116.

[7] K. Temming, R. M. Schiffelers, G. Molema, R. J. Kok, *Drug Resistance Updates* **2005**, *8*, 381–402.

[8] S. Liu, *Mol Pharm* **2006**, *3*, 472–487.

[9] P. G. Kyrtatos, P. Lehtolainen, M. Junemann-Ramirez, A. Garcia-Prieto, A. N. Price, J. F. Martin, D. G. Gadian, Q. A. Pankhurst, M. F. Lythgoe, *JACC Cardiovasc Interv* **2009**, *2*, 794–802.

[10] R. Asmatulu, A. Fakhari, H. L. Wamocha, H. Y. Chu, Y. Y. Chen, M. M. Eltabey, H. H. Hamdeh, J. C. Ho, *J Nanotechnol* **2009**, *2009*, 1–6.

[11] A. Schmidt, S. Bekeschus, K. Wende, B. Vollmar, T. von Woedtke, *Exp Dermatol* **2017**, *26*, 156–162.

[12] O. Lademann, A. Kramer, H. Richter, A. Patzelt, M. C. Meinke, V. Czaika, K. D. Weltmann, B. Hartmann, S. Koch, *Skin Pharmacol Physiol* **2011**, *24*, 284–288.

[13] D. Breden, C. A. Idicheria, S. Keum, P. M. Najt, L. L. Raja, *IEEE Transactions on Plasma Science* **2019**, *47*, DOI 10.1109/TPS.2018.2882830.

[14] H. Zhang, Z. Xu, J. Shen, X. Li, L. Ding, J. Ma, Y. Lan, W. Xia, C. Cheng, Q. Sun, Z. Zhang, P. K. Chu, *Sci Rep* **2015**, *5*, 1–12.

[15] Z. Shahbazi Rad, F. Abbasi Davani, *Clin Plasma Med* **2016**, *4*, 56–64.

[16] F. Kogelheide, K. Kartaschew, M. Strack, S. Baldus, N. Metzler-Nolte, M. Havenith, P. Awakowicz, K. Stapelmann, J.-W. Lackmann, *J Phys D Appl Phys* **2016**, *49*, 084004.

[17] C. Klinkhammer, C. Verlackt, D. Śmiłowicz, F. Kogelheide, A. Bogaerts, N. Metzler-Nolte, K. Stapelmann, M. Havenith, J. W. Lackmann, *Sci Rep* **2017**, *7*, 1–11.

[18] D. Śmiłowicz, F. Kogelheide, K. Stapelmann, P. Awakowicz, N. Metzler-Nolte, *Sci Rep* **2019**, *9*, 18024.