

**DEVELOPMENT OF BIOCONJUGATES AND THEIR
MODUL CONSTRUCTS FOR TARGETED THERAPY
OF CANCERS WITH HIGH MORTALITY**

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Effect of ionization conditions in electrospray ionization mass spectrometry of daunorubicin-tuftsins peptide conjugates

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Introduction

Daunomycin (Dau) is an anthracycline anticancer antibiotic that can bind to the DNA in the nucleus and can inhibit the topoisomerase II α enzyme.¹ The use of daunorubicin in clinical treatments is, however, limited by cardiotoxicity and other severe side-effects. Peptide-drug conjugates (PDCs) containing daunomycin for targeted therapy have been in the focus of intensive interest since years. These constructs are promising candidates to overcome clinical drawbacks. In PDCs, the drug moiety is attached to a targeting peptide, which can bind specifically to a receptor overexpressed on tumor cells, resulting in specific antitumor effect. Tuftsins derivatives have already been successfully applied in the development of drug delivery systems.^{2,3} Tuftsins (TKPR) is a natural tetrapeptide, which is a proteolytic fragment of the IgG Fc heavy chain.⁴ In this molecule, the lysine side chain provides an optimal coupling site for conjugation of drug molecules.

Mass spectrometry is a key technique for the fast and reliable identification of the products during the chemical synthesis of PDCs. However, in the case of anthracyclines and anthracycline derivatives, it is difficult to estimate the purity of the compounds due to an unusual fragmentation during MS analysis. Mass spectra of daunomycin containing bioconjugates show significant in-source fragmentation under the commonly used electrospray ionization mass spectrometry (ESI-MS). The main fragmentation pathway is the cleavage of the glycosidic bond. Emerging number of research papers are published in the field of novel anthracycline-containing PDCs.⁵⁻⁸ However, analytical data report complex mass spectra showing a mixture of protonated molecules, adduct ions and various fragment ions, and publications lack proper assignment and discussion of the detected peaks.

Our research was focused on the analysis of daunorubicin-containing PDCs using ESI-MS and on the determination of appropriate circumstances for the characterization of these complex molecules.⁹ Novel tuftsins-based bioconjugates were synthesized (Figure 1) to

investigate the quality of the mass spectra. The influence of the structure was studied, including the i) number of drug molecules; ii) number of basic functional groups; iii) presence or absence of an enzyme-labile spacer (GFLG) between the targeting peptide and the drug molecule.

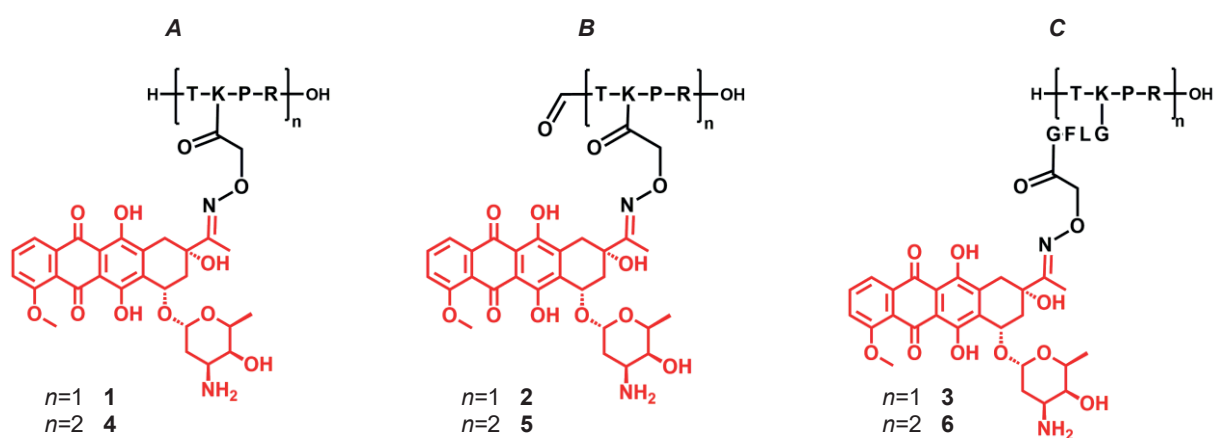


Figure 1. Schematic structure of novel tuftsin bioconjugates containing one or two daunomycin molecules with A.) free *N*-terminal amino group, B.) formylated *N*-terminus, C.) bearing a GFLG spacer.

Results

All peptides were synthesized by solid-phase methodology using Fmoc/tBu strategy. Daunomycin was conjugated to purified aminooxyacetylated peptides by oxime linkage. Tuftsin-daunorubicin bioconjugates and their dimer derivatives (containing two tuftsin units and thereby two daunomycin moieties (Figure 1) were synthesized and purified. Mass spectrometric experiments were performed on a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer equipped with electrospray ionization source, operating with continuous sample injection.

Commonly used ESI-MS experimental conditions for peptides is based on a solvent mixture composed of acetonitrile and water containing 0.1% acetic acid or formic acid. However, the mass spectra of these PDCs showed several peaks under these conditions. Fragment ions can be identified with high intensity, while intact protonated ions have lower abundance. Especially bioconjugates containing two daunomycin molecules (**4**, **5**, **6**) produced highly complex mass spectra showing a combination of sugar losses. We observed that a free *N*-terminal amino group facilitates the cleavage of the glycosidic bond, while blocking the *N*-terminal by formylation (**2**, **5**) results in lower fragmentation. Results indicate that incorporation of a neutral spacer (GFLG) between the peptide and the drug moiety (**3**, **6**), hereby moving the sugar moiety away from the basic tuftsin peptide backbone, slightly decreases the in-source fragmentation of the compounds.

We investigated the effect of the ion source tune parameters and the composition of the solvents used for the ionization of the samples as well. The results showed that significant reduction of the capillary exit potential (to 5 V) could slightly lower the fragmentation and increase the intensity of the intact protonated molecules. Other ion source parameters did not affect the ion ratios significantly.

Furthermore, we studied the effect of solvent mixtures while keeping the reduced capillary exit potential. Besides the commonly used acidic solvent, we used, for example, acetonitrile-water (50:50%, v/v) mixture and solutions containing ammonium bicarbonate (NH_4HCO_3 , 50 mM, pH 7.8) or ammonium acetate buffers (NH_4OAc , 50 mM, pH 6.7) and acetonitrile (50:50%, v/v). These neutral buffers can reduce the charge state of the ions. Our results show that the decrease in the number of charges on the protonated molecules formed during ESI ionization reduced the spontaneous dissociation of the glycosidic bond significantly.

The non-acidic acetonitrile-water mixture could decrease the amount of the fragment ions in case of conjugates **1**, **2**, **3** and **5**, while it was not effective for compounds **4** and **6**. However, in the case of the volatile buffers, intact protonated molecules of all bioconjugates were dominant. NH_4OAc buffer provided clearer ESI-MS spectra and over 95% intact protonated molecules (Figure 2). The best quality spectra could be achieved with a combination of low capillary exit potential (5 V) and NH_4OAc buffer (50 mM, pH 6.7).

Our results show that a higher number of positively charged functional groups in the PDC molecule indicated higher in-source fragmentation. Consequently, structural modifications, such as formylation can influence (enhance or decrease) the gas-phase stability of the molecules. We also found that enhanced distance between the sugar moiety and the positively charged peptide backbone decreases the fragmentation. The spontaneous dissociation of the glycosidic bond is facilitated by the highly charged peptide chain, therefore, shifting the charge states to lower charges can help to keep ions intact. Hence, application of neutral or slightly basic volatile buffers can significantly reduce the fragmentation of the analyte. In our experiments, the most appropriate buffer for suppressed fragmentation was ammonium acetate.

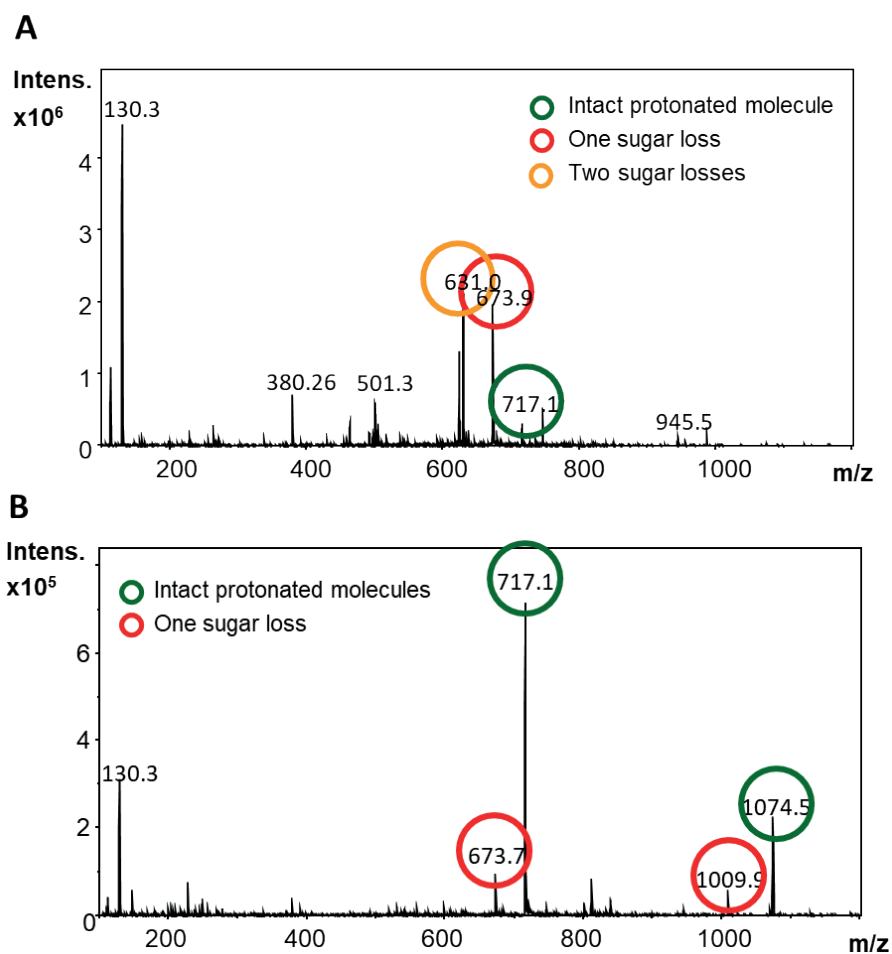


Figure 2. ESI-MS spectra of conjugate 4 in solvent mixtures containing *A.*) 0.1% acetic acid and *B.*) 50 mM NH_4OAc buffer, and acetonitrile (50:50, v/v). Capillary exit potential was set to 5 V in the case of spectrum *B.*

In conclusion, not only the settings of the mass spectrometer but also the structure of the daunorubicin-tuftsins conjugates had a high impact on the ESI-MS spectra. Conditions suggested here can be useful in the analysis of anthracycline-containing bioconjugates, to obtain mass spectra comprising intact protonated molecules.

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