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# The pivotal role of reactivity in the design of novel biotinylation reagents for the chemical-proteomics-based identification of vascular accessible biomarkers

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ABSTRACT

A promising approach for the development of novel therapeutics with fewer side effects in healthy tissues is the targeted delivery of bioactive molecules directly to the site of disease. Thus, one prerequisite is the identification of a robust, disease-specific, vascular accessible biomarker localized on the surface of diseased cells, in the surrounding extracellular matrix or on newly formed blood vessels. One avenue towards the identification of such biomarkers consists in the enrichment of the vascular accessible surface proteome fraction prior to analysis. This can be achieved by covalent modification of the target proteins with membrane-impermeable ester derivatives of biotin, followed by streptavidin-based affinity capturing. The properties of the respective reagents are determined by the linker between the biotin moiety and the reactive group for protein coupling. In the frame of this study, novel, reactivity-improved peptide-based biotinylation reagents as well as reagents based on highly hydrophilic heparin linkers were synthesized and validated. The comprehensive evaluation of different biotinylation reagent classes with aliphatic, PEGylated, peptide-based and heparin-based linkers on single model protein BSA, HeLa cells as well as perfused kidney tissue revealed that the linker-dependent chemical reactivity is the crucial factor for the design of novel biotinylation reagents for vascular targeting approaches.

*Significance:* To obtain a reliable identification and stable quantification of vascular accessible protein targets by means of mass spectrometry, covalent modification with a membrane-impermeable ester derivative of biotin, followed by streptavidin-based affinity capturing, is frequently applied for *in vivo* or *ex vivo* biomarker identification studies. Nevertheless, no comprehensive evaluation of different biotinylation reagent classes has been performed so far. Within this study, we systematically evaluated novel peptide- and heparin-based biotinylation reagents as well as established compounds based on aliphatic and PEGylated linkers. We identified the linker-dependant chemical reactivity of biotinylation reagents to be the critical factor for the design of novel reagents with high efficiency. The novel, site-specifically activated peptide-based reagents were found to be efficient compounds for application in mass spectrometry-based discovery of novel vascular-accessible biomarkers.



## 1. Introduction

The identification of vascular accessible, disease-specific biomarkers is a prerequisite for the development of novel antibody-based diagnostics or targeted therapies. Thereby, the molecules of interest are proteins expressed on the cell surface of diseased cells, on endothelial cells of newly formed blood vessels in the diseased tissue or in their perivascular extracellular matrix [1,2]. Global proteomic approaches for the identification of novel biomarkers with such an expression pattern have to deal with low expression levels of the highly heterogeneous surface proteome fraction as well as with the challenge of identifying hydrophobic plasma membrane proteins [3,4]. Even more important, not all surface exposed proteins within a diseased tissue can be reached by an antibody conjugate: Immunofluorescence studies with various tumor antigen-specific antibodies revealed a tissue penetration depth around blood vessels of only a few cell layers [5,6]. Reliable identification and quantification of novel, vascular accessible targets can be significantly facilitated by labeling and enrichment of the proteome fraction of interest prior to analysis. One approach is the vascular perfusion of rodents with membrane-impermeable, reactive

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ester derivatives of biotin, covalently reacting with the desired target proteome fraction [1,7]. The enriched vascular accessible proteome derived from diseased tissues and healthy organs can be subjected to comparative mass spectrometric analysis to identify novel disease-specific biomarkers [8–10]. The vascular perfusion technique can also be applied *ex vivo via* cannulation of the arteries of surgically resected human tissue material [11,12].

Biotinylation reagents used for vascular labeling of the target proteome fraction are composed of a biotin residue for affinity-based purification, a linker and a functional group for reaction with proteins. The complex formed by biotin and streptavidin homotetramers exhibits a very strong interaction (Kd ~ $10^{-15}$ M) and can therefore be exploited for capturing of the biotinylated target proteins out of protein extracts on streptavidin resin before proteomic analysis [13]. Proteins are composed of a variety of functional groups exploitable for chemical derivatization. For successful modification of the whole cell surface proteome fraction, the targeted functional group has to be expressed in an accessible form on the cell surface. Therefore, reactive ester derivatives of biotin targeting ε-amines in lysine side chains or the protein N-terminus in a one-step reaction are widely applied for vascular labeling [14]. The linker between the biotin moiety for affinity purification and the reactive group for protein modification affects the properties of a biotinylation reagent. The linker serves as spacer to minimize steric hindrance during the chemical reaction with protein amino groups and throughout the capturing process. In order to allow for the biotinylation of proteins in living cells, the reagent has to be soluble in aqueous buffers at a concentration of at least 1 mg/ml [15]. Furthermore, the reagent must not cross biological membranes due to the localization of potential vascular accessible biomarkers. Physicochemical characteristics of biotinylation reagents can be changed by increasing the linker's size and the number of charges [15]. In 2010, the biotinylation reagent NHS-β-Ala-(L-Asp)$_3$-biotin was published, exhibiting three negatively charged aspartic acid side chains, and therefore increased water solubility, as well as a 36% increase in size compared to commercial Sulfo-NHS-LC-biotin [15]. The comparative proteomic analysis of perfused murine kidney tissue resulted in a total of 219 surface annotated proteins and revealed a slight up-regulation of surface or extracellular matrix proteins with the membrane-impermeable, peptide-based reagent.

Within this study, different biotinylation reagent classes based on novel peptide- and heparin-linkers were designed and evaluated in comparison to commercial reagents based on aliphatic or PEGylated linkers. The efficiency of novel, site-specifically activated peptide-based biotinylation reagents was thereby evaluated in comparison to NHS-β-Ala-(L-Asp)$_3$-biotin. Furthermore, novel biotinylation reagents based on a multiply negatively charged heparin linker were designed to examine the effect of large size spacers on the reagents' reactivity.

## 2. Materials & methods

Detailed information on reagent synthesis, mass spectrometric methods and label-free quantification of mass spectrometric data can be found in the Supplementary material.

### 2.1. Materials

If not stated otherwise, reagents were purchased from Sigma-Aldrich, solvents in ULC/MS grade from Biosolve. The commercial biotinylation reagents Sulfo-NHS-LC-biotin (ProteoChem) and NHS-PEG$_{12}$-biotin (Thermo Scientific) were used for validation purposes.

#### 2.1.1. Synthesis of peptide-based biotinylation reagents

Synthesis of the biotinylated peptides Biotin-(L-Asp)$_3$-β-Ala-OH and Biotin-(L-Asp)$_3$-Cys-CONH$_2$ was performed on an Intavis peptide synthesizer using solid phase peptide synthesis with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Activation of Biotin-(L-Asp)$_3$-β-Ala-OH with *N*-hydroxysuccinimide (ProteoChem) was performed directly before use. Biotin-(L-Asp)$_3$-Cys-CONH$_2$ was coupled to either succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC crosslinker, ProteoChem) or succinimidyl-([*N*-maleimidopropionamido]hexaethyleneglycol)ester (SM(PEG)$_6$ crosslinker, Thermo Scientific) directly before use. In the following, the activated reagents are referred to as NHS-β-Ala-(L-Asp)$_3$-biotin, SMCC-Cys-(L-Asp)$_3$-biotin and SM(PEG)$_6$-Cys-(L-Asp)$_3$-biotin.

#### 2.1.2. Synthesis of heparin-based biotinylation reagents

Heparin sodium salt from porcine intestinal mucosa (Calbiochem) and low-molecular weight enoxaparin sodium (Dongying Tiandong Biochemical Industry) were biotinylated by introduction of biotin hydrazide (ProteoChem) at the reducing chain ends. sNHS-activation of carboxylic acid side chains was performed with *N*-hydroxysulfosuccinimide (sNHS, ProteoChem) directly before use. In the following, the activated reagents are referred to as Biotin-Heparin-sNHS and Biotin-Enoxaparin-sNHS.

#### 2.1.3. Reactivity assessment

10 mM biotinylation reagent stock was prepared in water directly before use. The reagent solution was added to 100 μg bovine serum albumin (BSA) aliquots (1.505 nmol, 1 eq, 1 mg/ml) in phosphate buffered saline (PBS) in different ratios (1 eq/1.505 nmol–64 eq/96.3 nmol). The reaction was quenched after 15 min incubation at room temperature using ten-fold excess of Tris solution (100 mM in water). Following acidification to a final concentration of 0.1% trifluoroacetic acid (TFA, ProteoChem), samples were desalted using OMIX C4 pipette tips (Agilent Technologies) and dried in a vacuum concentrator. Samples were resolubilized in 5% acetonitrile, 0.1% TFA in water and co-crystalized with sinapinic acid (ProteoChem) onto a MALDI target plate. Mass spectrometric analysis was performed on a MALDI TOF/TOF™ 5800 system (Sciex) in linear positive mode. MS spectra were analyzed using the Data Explorer® Software (version 4.10, Applied Biosystems). The mass of the biotinylated protein *M*(*BSA modified*) was extracted at the maximum of the singly charged peak. The number of reagent tags *N* added per BSA molecule was calculated using the mass of unmodified, singly charged BSA *M*(*BSA*) and the mass of the corresponding reagent tag *M*(*reagent*) according to Eq. 1.

$$N = \frac{M\,(BSA\ modified) - M\,(BSA)}{M(reagent)} \quad (1)$$

#### 2.1.4. In vitro biotinylation of cells

HeLa cells were cultured in IMDM medium (Gibco) supplemented with 10% FCS (PAN Biotech) at 37 °C and 5% $CO_2$. Cell line authenticity was confirmed by SNP-profiling of extracted DNA (Multiplexion GmbH, Heidelberg, Germany). For reactivity assessment *via* fluorescence-activated cell sorting (FACS analysis), cells were non-enzymatically detached in 10 mM ethylenediaminetetraacetic acid (EDTA) in PBS and aliquoted prior to biotinylation. 10 mM biotinylation reagent stock was prepared in water directly before use. $1 \times 10^6$ cells were incubated with different ratios from 0.06 nmol to 60 nmol of biotinylation reagent. After 5 min, the reaction was quenched with a ten-fold excess of 0.1 M Tris solution in PBS. Cells were pelletized at 300 ×*g* and resuspended in 2% FCS in PBS for FACS analysis. Negative control cells were kept in PBS. For mass spectrometric analysis, $4 \times 10^6$ to $5 \times 10^6$ adherent cells were washed twice with PBS and incubated with 5 μmol biotinylation reagent in 5 ml PBS for 5 min at 37 °C. After quenching with a ten-fold excess of 0.1 M Tris solution in PBS, cells were washed twice with PBS and non-enzymatically detached in 10 mM EDTA in PBS. After pelletizing, biotinylated HeLa cells were lysed in 2 ml RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in water) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) for mass spectrometric analysis. Cell lysis was completed by