# The Multifaceted Role of Glycosaminoglycans in the Cellular Uptake of Cell-Penetrating Peptides

R. Wallbrecher[1], S. Schmidt[1], W. P. Verdurmen[1], P. H. Bovee-Geurts[1], F. Broecker[2,3], A. Reinhardt[2,3], T. H. van Kuppevelt[1], J. Eilander[1], S. Fanghänel[4], J. Bürck[5], P. Wadhwani[5], P. H. Seeberger[2,3], A. S. Ulrich[4,5], R. Brock[1]

[1]Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, 6525 GA Nijmegen, the Netherlands; [2]Department of Biomolecular Systems, Max-Planck-Institute of Colloids and Interfaces, 14476 Potsdam, Germany; [3]Institute of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany; [4]Karlsruhe Institute of Technology (KIT), Institute for Organic Chemistry and CFN, 76131 Karlsruhe, Germany; [5]KIT, Institute for Biological Interfaces (IBG-2), 76021 Karlsruhe, Germany
r.brock@ncmls.ru.nl

## ABSTRACT SUMMARY

Here, we demonstrate that in spite of a general propensity to interact with CPPs the role of GAG in uptake varies greatly and depends on the nature of the CPP. For variants of a CPP derived from the human lactoferrin protein there was a negative correlation of the stoichiometry of GAG binding and uptake, indicating that GAG clustering promoted uptake. This CPP shares characteristics with other arginine rich CPP such as nonaarginine. In contrast, for analogs of the amphipathic transportan 10 (TP10) clustering impeded uptake. To monitor GAG clustering in living cells, metabolic labeling of sugars was employed. Here, cells are incubated with azido-bearing sugar analogs. Following incorporation into sialic acids, fluorescent labels can be introduced by click chemistry

## INTRODUCTION

Cationic CPPs hold great promise as delivery vectors for a wide variety of cargo. However, the molecular mechanisms of uptake are still not fully clear. The interaction with negatively charged glycosaminoglycans at the cell surface has been broadly associated with the uptake of cationic CPP[1]. In particular, it was proposed that clustering of GAG and GAG-bearing proteins such as syndecans leads to activation of actin reorganization and induction of endocytosis[2]. Here, we asked how general this concept applies across classes of cationic CPP, employing variants of the human lactoferrin-derived CPP and variants of the amphipathic CPP TP10.

## EXPERIMENTAL METHODS

hLF variants were purchased from EMC microcollections (Tübingen, Germany), TP10 variants were synthesized by Fmoc chemistry. All peptides beared an N-terminal carboxy-fluorescein moiety. HeLa cells were incubated at 37°C with the indicated peptide concentrations. Uptake efficiencies were quantified by flow cytometry and the cellular peptide distribution and GAG clustering visualized by confocal microscopy. To determine the thermodynamic characteristics of the heparan sulfate (HS)-CPP interaction, isothermal titration calorimetry (ITC) was performed using an ITC200 Microcal. HS was injected into peptide solutions; concentrations were adapted for the different hLF variants in order to ensure a high signal-to-noise ratio. Measurements were performed in HBS at 25 °C.

**Table 1.** Sequences of peptides used in this study. Residues that differ from the parent sequences are given in bold face.

| Name | Sequence |
|---|---|
| TP10 | Fluo-AGYLLGKINLKALAALAKKIL-$NH_2$ |
| TP10 2GL | Fluo-A-***L*-CF3-Bpg**-YLLGKINLKALAALAKKIL-$NH_2$[1] |
| hLF | Fluo-KCFQWQRNMRKVRGPPVSCIKR-$NH_2$ |
| hLF R7 | Fluo-**R**CFQWQRNMR**R**VRGPPVSCI**R**R-$NH_2$ |
| hLF +4R | Fluo-KCF**R**WQRN**R**RKVRG**R**PV**R**CIKR-$NH_2$ |
| hLF lin +4R | Fluo-K**R**F**R**WQRNMRKVRG**R**PV**R**CIKR-$NH_2$ |
| hLF Hcy | Fluo-K-**Hcy**-FQWQRNMRKVRGPPVS-**Hcy**-IKR-$NH_2$[2] |

[1]L-CF3-Bpg corresponds to the *L*-enantiomer of trifluoromethyl-bicyclopent-[1.1.1]-1-ylglycine, a spin label for solid-state NMR. [2]Hcy: homocysteine

HS-induced clustering of TP10 variants in solution was determined by dynamic light

scattering (DLS) using a Zetasizer Nano S (Malvern Instruments, Malvern, UK) at a peptide concentration of 5 μM and an HS concentration of 10 μM. To visualize TP10-induced GAG clustering on living cells metabolic sugar labeling using copper-free click chemistry was performed[3].

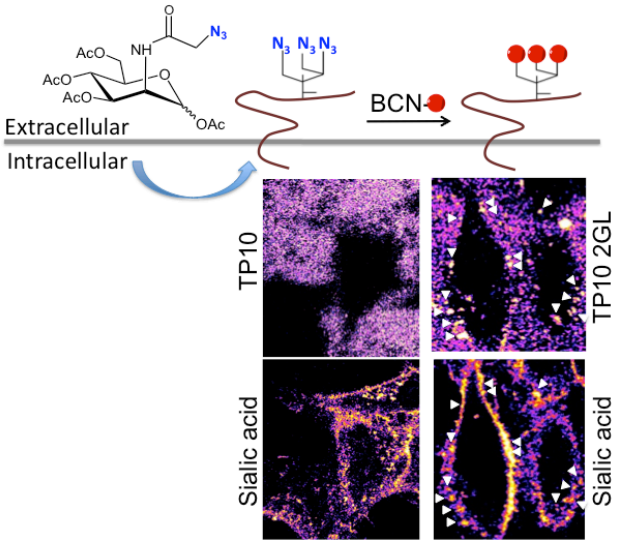


**Figure 1.** Monitoring of GAG clustering by metabolic labeling of sialic acids with BCN (Bicyclo [6.1.0] nonyne) a reagent for copper-free click chemistry. Cells were incubated with peptides (5 μM) in the presence of serum for 30 min.

## RESULTS AND DISCUSSION

When investigating the uptake efficiency of a series of TP10 variants, containing a solid-state spin label, we noticed that the variant TP10 2GL had a cellular association that was 2.5 times that of the parent peptide[4]. However, uptake, as determined by trypan blue incubation of cells which quenches fluorescence of peptides associated to the outside of cells, was the same as for the parent peptide[4]. DLS measurements demonstrated that in solution the 2GL variant had an increased capacity to cluster HS. In order to investigate whether clustering also occurred on the cell surface, we employed metabolic labeling of sialic acids, which allows monitoring of GAG clustering without the need to express GFP-fusion proteins or immuno-labeling. Also on cells, the variant strongly induced GAG clustering (Figure 1). In contrast, variants of the hLF CPP, which differed in the number and positions of arginines and the nature of the disulfide bond, showed a strong negative correlation of the stoichiometry of HS binding and uptake which can be best explained by an uptake mechanism in which GAG clustering promotes uptake[5].

## CONCLUSION

The comparison of TP10 and hLF variants demonstrates that no general mechanism for GAG-dependent uptake exists and the role of GAG is a function of the particular type of CPP. It should be possible to exploit this difference in GAG-dependence for a cell-type specific targeting, where those peptides for which uptake is promoted by GAG show preferential uptake by GAG expressing cells and vice versa.

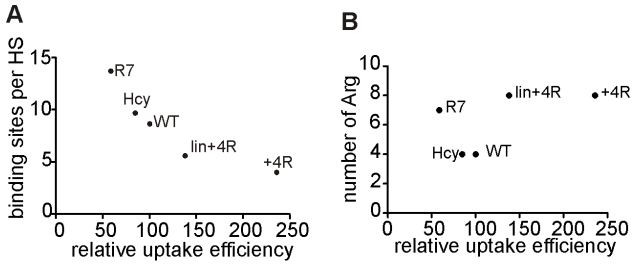


**Figure 2.** Correlation of uptake of hLF variants with structural and thermodynamic parameters. Binding sites were derived from ITC measurements at individually adjusted peptide concentrations. Cellular uptake was measured at 5 μM.

## REFERENCES


1. Nakase I, et al. *Biochemistry* 46 (2007) 492-501.
2. Letoha T, et al. *Biochim Biophys Acta* 1798 (2010) 2258-2265.
3. Dommerholt J, et al. *Angew Chem Int Ed Engl* 49 (2010) 9422-9425.
4. Verdurmen WP, et al. *J Control Release* 170 (2013) 83-91.
5. Wallbrecher R, et al. *Cell Mol Life Sci*. (2013) PMID: 24270856


## ACKNOWLEDGMENTS


We thank Mattias Hällbrink for helpful discussions. W.P.R.V. was supported from funds of the Radboud University Nijmegen Medical Centre, R.W. was supported by BMBF Biotransporter (13N11454), S.S. by funds from the Roche postdoc programme, A. R. from the Studienstiftung des Deutschen Volkes and the Max-Planck-Society. A.S.U. acknowledges the DFG-Center for Functional Nanostructures (TP E1.2).