Chapter 8

Methods to Study the Role of the Glycocalyx in the Uptake of Cell-Penetrating Peptides

Samuel Schmidt, Rike Wallbrecher, Toin H. van Kuppevelt, and Roland Brock

Abstract

Cells are covered by a layer of negatively charged oligo- and polysaccharides, the glycocalyx. Cell-penetrating peptides and other drug delivery vehicles first encounter these polyanions before contacting the lipid bilayer of the plasma membrane. While a large body of data supports the notion that interactions with the glycocalyx promote or even trigger uptake, in some cases, the glycocalyx compromises delivery. As a consequence there is a need to address the role of the glycocalyx in delivery for each specific delivery vehicle and for each particular type of cell. Here, we describe protocols to obtain information on the composition and dynamics of the glycocalyx, and the role of individual glycocalyx components in the uptake of drug delivery vehicles.

Key words Click chemistry, Glycosaminoglycans, Immunofluorescence, Metabolic labeling, Single-chain antibody fragments

1 Introduction

Molecules approaching a cell first encounter a layer of oligo- and polysaccharides, the glycocalyx. The major components of the glycocalyx are proteoglycans which carry highly negatively charged repeating disaccharide units, the glycosaminoglycans (GAGs) [1]. Heparan sulfates, chondroitin sulfates, dermatan sulfates, and hyaluronic acid are important constituents of the glycocalyx. However, in the context of cell-penetrating peptide (CPP) uptake heparan sulfates have been studied most intensively [2, 3]. Furthermore, the terminal structures of the glycan moieties of glycoproteins and glycolipids can contain the negatively charged sialic acids.

Even though CPPs do not interact with specific receptors, a current model for the induction of endocytic uptake comprises the binding to heparan sulfates, followed by cross-linking of the associated proteoglycans which then leads to activation of small GTPases and subsequent actin reorganization [4]. Nevertheless,

Ülo Langel (ed.), *Cell-Penetrating Peptides: Methods and Protocols*, Methods in Molecular Biology, vol. 1324,
DOI 10.1007/978-1-4939-2806-4_8,

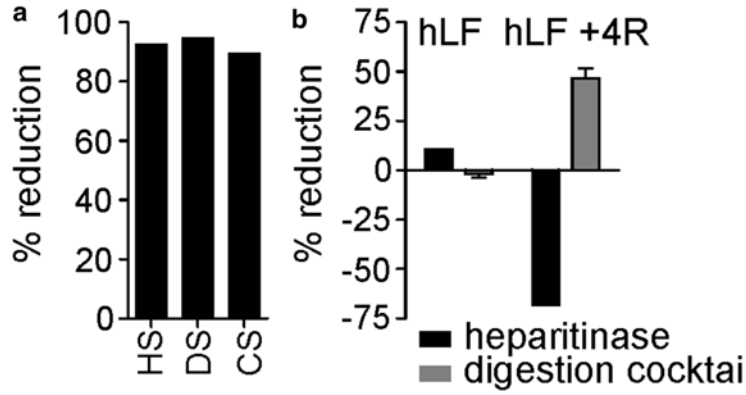

Fig. 1 Effect of enzymatic digestion of the glycocalyx on CPP uptake. (**a**) Assessment of enzymatic GAG removal by immunofluorescence. The percentage of reduction was calculated by comparing the signal as determined by flow cytometry before and after digestion. HS refers to median fluorescence obtained for the single-chain antibody HS4C3, CS to I03H10V and DS to LKN1V. Data of one single experiment are shown. (**b**) Degree of reduction in peptide uptake after heparitinase I treatment or glycocalyx removal by the enzyme cocktail. Heparitinase samples show a single experiment. For full digestion, $n=2$. Error bars show SEM. Peptides were N-terminally labeled with carboxyfluorescein (Fluo) and amidated at the C-terminus (hLF—Fluo-KCFQWQRNMRKVRGPPVSCIKR-NH_2, hLF + 4R—Fluo-KCFRWQRNRRKVRGRPVRCIKR-NH_2, where -NH_2 stands for the C-terminal amidation) [15]. A negative reduction indicates an increase in peptide uptake.

a number of observations do not fit into this concept (reviewed in Favretto et al. [2]). For example, using a cell line which is deficient in specific types of GAGs, it was found that the uptake of the Tat peptide was promoted [5].

Therefore, further analyses of the role of GAGs in uptake are urgently needed. In addition, a refinement of experimental protocols is required. Since cells defective in GAG synthesis [6–8] may potentially show long-term compensation, for the analysis of GAG involvement the use of protocols to analyze and manipulate GAG levels for a given cell type are desirable. At this point, most studies have only addressed heparan sulfates, even though this glycosaminoglycan may only comprise a minor part of the total GAG layer and thus removal of HS alone will also have only a minor effect on uptake, compared to total GAG removal (Fig. 1). We will provide protocols for a complete digestion of the glycosaminoglycans. In addition, we describe immunofluorescence protocols using a collection of single-chain antibody fragments to control for GAG chain removal and also compare cells with respect to GAG expression. Metabolic labeling is described as a means to label sialic acids which are hard to capture using immunofluorescence. The latter

Table 1
Overview of the presented methods

Method	Reagent	Aspect studied
Bioorthogonal labeling	Ac4ManNAz, BCN-biotin, Streptavidin-AF 488	Sialic acid distribution, GAG clustering, effectiveness of enzymatic removal
Immunofluorescence	HS4C3, EV3C3, A04B08	Heparan sulfate (relative expression level/distribution/completeness of digestion)
Immunofluorescence	I03H10	Chondroitin sulfate (relative expression level/distribution/completeness of digestion)
Immunofluorescence	GD3A12, LKN1	Dermatan sulfate (relative expression level/distribution/completeness of digestion)
Enzymatic removal	Heparitinase I, Chondroitinase ABC, Hyaluronidase, Neuraminidase	Involvement in CPP uptake

also provides the means to follow dynamics of the glycocalyx in living cells without the need to introduce genetically tagged fusion proteins. In combination, these methods provide detailed insights into the involvement of GAGs in uptake (Table 1).

2 Materials

For performing the protocols on GAG analysis described below no adjustments of tissue culture protocols are required. Cells can be propagated according to the requirements of each particular cell line. Therefore, no specifications for tissue culture reagents and protocols are provided.

2.1 Single-Chain Antibody Fragments and Reagents for Indirect Immunofluorescence

1. Anti-HS single-chain antibodies (HS4C3, A04B08, EV3C3) [9–11], anti-DS single-chain antibodies (LKN1, GD3A12) [12, 13], and the anti-CS single-chain antibody (I03H10) [14]. Except for cases in which purity is required, periplasmic fractions are used. All single-chain antibodies carry a VSV-tag for recognition by a secondary antibody.
2. Second mouse-anti VSV monoclonal antibody (P5D4) is from hybridoma culture supernatant. Cell line (P5D4) is available from American Type Culture Collection (ATCC). Alternatively, polyclonal rabbit anti-VSV-G (Sigma) can be used.
3. Goat-anti-mouse IgG Alexa Fluor 488-conjugated antibody (Invitrogen, Life Technologies, Carlsbad, USA).
4. Hepes-buffered saline (HBS; 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4).

2.2 Detection of GAG Chains and Sialic Acids on HeLa Cells Using Live Cell Confocal Microscopy

1. 8-well microscopy chambers (Nunc, Wiesbaden, Germany).
2. TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany), equipped with an HCX PL APO 63×N.A. 1.2 water immersion lens with 37 °C incubation chamber.
3. Fiji ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, USA) for image analysis.

2.3 Detection of GAG Chains and Sialic Acids on HeLa Cells Using Flow Cytometry

1. 2 mM EDTA (PAN Biotech, Aidenbach, Germany) in HBS.
2. Propidium iodide (Sigma-Aldrich, St. Louis, USA).
3. FACS tubes (Micronic, Lelystad, the Netherlands).
4. FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium).
5. DeNovo FCS 4.0 (De Novo Software, Glendale, USA) and Summit 4.3 (Dako, Fort Collins, USA).

2.4 GAG-Digesting Enzymes

1. Heparitinase I (Seikagaku, Tokyo, Japan).
2. Hyaluronidase (Sigma Aldrich, St. Louis, USA).
3. Chondroitinase ABC (*see* **Note 1**; Sigma Aldrich, St. Louis, USA).
4. Neuraminidase (Sigma Aldrich, St. Louis, USA).

2.5 Reagents for Metabolic Labeling

1. Peracetylated N-azidoethylmannosamine (Ac4ManNAz, 50 mM stock in milliQ, SynAffix, Nijmegen, the Netherlands). Store at −20 °C.
2. Biotin-conjugated Bicyclo [6.1.0] nonyne (BCN, 10 mM stock in MillQ, SynAffix, Nijmegen, the Netherlands). Store at −20 °C.
3. Alexa Fluor 647-conjugated streptavidin (Invitrogen, Life Technologies, Carlsbad, USA). Store at −20 °C.
4. HBS.

3 Methods

3.1 Detection of Cell Surface GAG Chains on Live Cells

1. Grow HeLa cells (ATCC) in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10 % FCS and 2 mM L-glutamine.
2. One day before the experiment seed 40,000 HeLa cells in 8-well microscopy chambers yielding a confluency of approximately 80 %.
3. Remove medium and wash adherent cells gently once with HBS.
4. Prepare the antibody solutions in HBS (dilution of antibodies against HS epitopes 20 times, of antibodies against DS and CS 5 times) (*see* **Note 2**). Transfer the solution gently on top of the adherent cells (*see* **Note 3**). Prepare 150 µL per well.

5. Incubate cells for 45 min at 20 °C.
6. Remove the incubation solution and wash the adherent cells three times with HBS.
7. Dilute the second antibody, mouse-anti VSV (P5D4), ten times in HBS. Use HBS as a control.
8. Incubate cells for 30 min at 4 °C (*see* **Note 4**).
9. Remove the antibody solution and wash cells three times with HBS.
10. Prepare the third antibody, goat-anti-mouse IgG Alexa Fluor 488 in HBS at a final concentration of 10 μg/mL (or HBS as control). Conjugates with other fluorophores can be used as desired/needed by the specific experimental condition.
11. Incubate cells for 30 min at 4 °C.
12. Remove the incubation solution.

For live cell confocal microscopy:

13. Wash the cells three times with pre-warmed culture medium.
14. Perform confocal microscopy using excitation at 488 nm and detection of fluorescence over 500–550 nm (*see* **Note 5**).

For flow cytometry:

13. Wash the cells three times with HBS.
14. Detach the cells using HBS-EDTA (*see* **Note 6**).
15. Transfer the cell suspension into FACS tubes.
16. Add propidium iodide at a final concentration of 2.5 μg/mL directly before the measurement. Measure fluorescence using flow cytometry. Measure Alexa Fluor 488 in the FL1 channel and propidium iodide in channel FL3 (*see* **Notes 5** and **7**).

3.2 Bioorthogonal Labeling of Cell Surface Sialic Acid Residues on Live Cells

1. Seed cells as in Subheading 3.1 (*see* **Note 8**).
2. Add peracetylated *N*-azidoacethylmannosamine at a final concentration of 50 μM to the cells. The sugar analog will enter the cells to be incorporated into the glycocalyx for direct monitoring of GAG clustering (Figs. 2 and 3).
3. Incubate cells overnight at 37 °C, 5 % CO_2.
4. Remove medium and wash cells gently with HBS three times.
5. Prepare a BCN-biotin solution in HBS with a final concentration of 60 μM (*see* **Note 9**). In control samples use MilliQ instead of BCN-biotin in HBS.
6. Incubate cells for 1 h at 20 °C.
7. Remove the incubation solution and wash cells three times with HBS.
8. Dilute Alexa Fluor 647-conjugated streptavidin to a final concentration of 5 μg/mL in HBS.

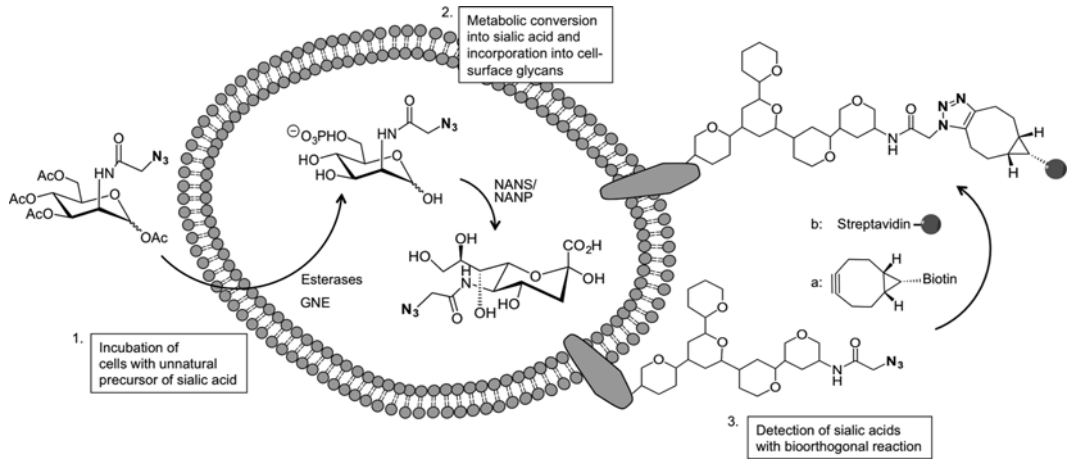

Fig. 2 Scheme of metabolic labeling. Exogenously added peracetylated *N*-azidoacethylmannosamine (Ac4ManNAz) can be taken up by cells and is deacetylated by intracellular esterases, and then enters into the salvage pathway, including UDP-GlcNAc 2-epimerase/ManNAc-6-kinase (GNE)-mediated phosphorylation of ManNAz and the generation of phosphorylated Neu5Ac (Neu5Ac-9-phosphate) by *N*-acetylneuraminic acid synthase (Neu5Ac-9-P synthetase/NANS) and dephosphorylation of Neu5Ac by *N*-acetylneuraminic-acid Phosphatase (Neu5Ac-9-P Phosphatase/NANP). After further modifications in the nucleus and the golgi apparatus, the azido-sialic acids appear as part of glycan moities of glycoproteins at the cell surface [16]. The detection of cell surface azido-sialic acids can be performed by using e.g. Bicyclo [6.1.0] nonyne (BCN) (*a*) in a metal-free strain promoted alkyne-azide cycloaddition (SPAAC) reaction, giving stable 1,2,3-triazoles. Finally, a fluorophore-conjugated streptavidin (*b*) can be used to visualize bioorthogonally labeled sialic acids using, e.g. confocal microscopy [17]

9. Incubate cells for 30 min at 4 °C.
10. Remove the incubation solution.
11. If peptide internalization is supposed to be measured, then the peptide solution should be added here.

For live cell confocal microscopy:

12. Wash the cells three times with pre-warmed culture medium.
13. Keep the cells for 1 h at 37 °C, 5 % CO_2 in culture medium before treatment with CPPs.
14. Perform confocal microscopy. Use the 633 nm laser line for excitation and collect the emission between 650 and 750 nm.

For flow cytometry:

15. Wash the adhered cells three times with HBS.
16. Detach the cells using HBS-EDTA.
17. Transfer the cell suspension to FACS tubes.
18. Measure the fluorescence using FACS-Calibur in the FL4 channel.

3.3 Enzymatic Removal of the Glycocalyx

1. In order to confirm the efficient removal of sialic acids, cells have to be fed with peracetylated *N-azidoacethylmannosamine* as described in Subheading 3.2 (bioorthogonal labeling)

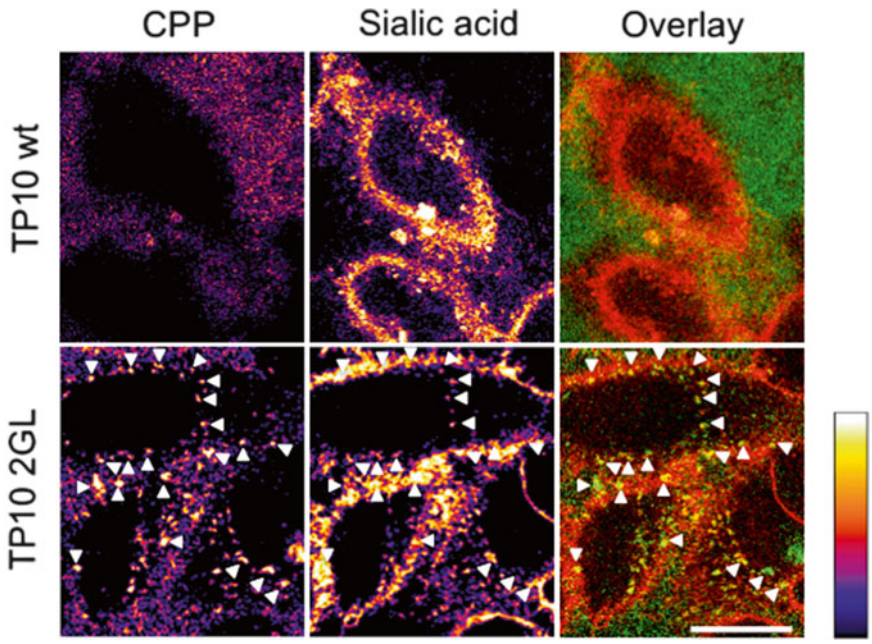

Fig. 3 Colocalization study of sialic acid with modified transportan peptides. Sialic acid moieties on HeLa cells were bioorthogonally labeled as described in Subheading 3.2. Subsequently, fluorescein-labeled TP10 wt or TP10 2GL (TP10 Gly2 → L-CF3-Bpg) was administered in a final concentration of 5 μM and confocal microscopy was performed after 30 min at 37 °C. Representative confocal images of cells are shown. For TP10 wt, no cluster formation was observed and no apparent changes in the cell surface distribution of sialic acids were visible (*upper panel*). In contrast, for TP10 Gly2 → L-CF3-Bpg, clusters are visible of cell surface sialic acid moieties which colocalizes with Gly2 → L-CF3-Bpg. Clusters are highlighted by *arrowheads* (*lower panel*). The false color look-up table ranges from *black* (low) to *white* (high) [18]. The scale bar corresponds to 20 μm

2. Seed cells as in Subheading 3.1
3. Prepare digestion cocktail containing all enzymes (Heparitinase I, Chondroitinase ABC, Neuraminidase, Hyaluronidase) or only some of the enzymes. When using hyaluronidase, weigh 2.75 mg hyaluronidase and add 5 mL RPMI + 1 % FCS yielding 275–550 U/mL.
4. If digestions of other GAGs are carried out in the presence of hyaluronidase take the required volume from the hyaluronidase mix (150 μL per sample) and add other enzymes. If hyaluronidase is not required add other enzymes to RPMI + 1 % FCS.
5. Add the other enzymes at the following concentrations: 10 mU/mL heparitinase I, 100 mU/mL chondroitinase ABC, 100 mU/mL neuraminidase.
6. Incubate cells for 1.5 h at 37 °C.
7. Wash cells with HBS twice.
8. Start peptide incubation as in Subheading 3.2 or stain glycocalyx as in Subheading 3.1.

4 Notes

1. Chondroitinase B is the dermatan sulfate-digesting enzyme.
2. No exact concentration of the scFvs can be measured as periplasmic fractions are used. Therefore, only dilution factors can be provided which may differ between batches.
3. In case of non-adherent or loose cells staining should be performed in suspension with EDTA-detached cells. Cells should be washed by centrifugation.
4. Incubation with the second antibody (P5D4) could also be performed at RT instead of 4 °C in order to reduce the stress for the cells.
5. If GAG labeling is to be performed concomitantly with uptake of fluorescein-labeled peptides, the third antibody comprising an alternative fluorophore such as Alexa Fluor 647 could be used.
6. Detachment of cells with 2 mM EDTA because protein cores of proteoglycans would be cleaved off if trypsin was used.
7. Text needs to be replaced by: Alexa Fluor 488 and fluorescein were detected in FL1 (530/30 BP), Propidium iodide in FL3 (< 670 LP) and excited with the 488 nm line of an argon ion laser. Alexa Fluor 647 was detected in FL4 (661/16 BP) and excited with the 647 nm line of a 635 nm-red diode laser.
8. For the analysis of sialic acid, it is important to cultivate the cells in RPMI and not in DMEM to prevent a strong competition between the *N*-azidoacethylmannosamine and the high glucose in DMEM.
9. Instead of using biotinylated BCN the bioorthogonal labeling can be performed using BCN which is directly conjugated to a fluorophore, e.g. BCN-Dy649 to prevent a possible crosslinking of the glycocalyx by streptavidin.

Acknowledgements

S. S. was supported by the Roche postdoc programme (ID 272).

References

1. Couchman JR (2010) Transmembrane signaling proteoglycans. Annu Rev Cell Dev Biol 26:89–114
2. Favretto ME, Wallbrecher R, Schmidt S, van de Putte R, Brock R (2014) Glycosaminoglycans in the cellular uptake of drug delivery vectors - bystanders or active players? J Control Release 180C:81–90
3. Christianson HC, Belting M (2014) Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. Matrix Biol 35:51–55

4. Gerbal-Chaloin S, Gondeau C, Aldrian-Herrada G, Heitz F, Gauthier-Rouviere C, Divita G (2007) First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling. Biol Cell 99:223–238
5. Subrizi A, Tuominen E, Bunker A, Rog T, Antopolsky M, Urtti A (2012) Tat(48-60) peptide amino acid sequence is not unique in its cell penetrating properties and cell-surface glycosaminoglycans inhibit its cellular uptake. J Control Release 158:277–285
6. Esko JD, Stewart TE, Taylor WH (1985) Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc Natl Acad Sci U S A 82:3197–3201
7. Esko JD, Weinke JL, Taylor WH, Ekborg G, Roden L, Anantharamaiah G, Gawish A (1987) Inhibition of chondroitin and heparan sulfate biosynthesis in Chinese hamster ovary cell mutants defective in galactosyltransferase I. J Biol Chem 262:12189–12195
8. Lidholt K, Weinke JL, Kiser CS, Lugemwa FN, Bame KJ, Cheifetz S, Massague J, Lindahl U, Esko JD (1992) A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. Proc Natl Acad Sci U S A 89: 2267–2271
9. van Kuppevelt TH, Dennissen MA, van Venrooij WJ, Hoet RM, Veerkamp JH (1998) Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney. J Biol Chem 273:12960–12966
10. Jenniskens GJ, Oosterhof A, Brandwijk R, Veerkamp JH, van Kuppevelt TH (2000) Heparan sulfate heterogeneity in skeletal muscle basal lamina: demonstration by phage display-derived antibodies. J Neurosci 20:4099–4111
11. Smits NC, Robbesom AA, Versteeg EM, van de Westerlo EM, Dekhuijzen PN, van Kuppevelt TH (2004) Heterogeneity of heparan sulfates in human lung. Am J Respir Cell Mol Biol 30:166–173
12. Ten Dam GB, Yamada S, Kobayashi F, Purushothaman A, van de Westerlo EM, Bulten J, Malmstrom A, Sugahara K, Massuger LF, van Kuppevelt TH (2009) Dermatan sulfate domains defined by the novel antibody GD3A12, in normal tissues and ovarian adenocarcinomas. Histochem Cell Biol 132: 117–127
13. Lensen JF, Wijnhoven TJ, Kuik LH, Versteeg EM, Hafmans T, Rops AL, Pavao MS, van der Vlag J, van den Heuvel LP, Berden JH, van Kuppevelt TH (2006) Selection and characterization of a unique phage display-derived antibody against dermatan sulfate. Matrix Biol 25:457–461
14. Smetsers TF, van de Westerlo EM, ten Dam GB, Overes IM, Schalkwijk J, van Muijen GN, van Kuppevelt TH (2004) Human single-chain antibodies reactive with native chondroitin sulfate detect chondroitin sulfate alterations in melanoma and psoriasis. J Invest Dermatol 122:707–716
15. Wallbrecher R, Verdurmen WP, Schmidt S, Bovee-Geurts PH, Broecker F, Reinhardt A, van Kuppevelt TH, Seeberger PH, Brock R (2014) The stoichiometry of peptide-heparan sulfate binding as a determinant of uptake efficiency of cell-penetrating peptides. Cell Mol Life Sci 71:2717–2729
16. Munster-Kuhnel AK, Tiralongo J, Krapp S, Weinhold B, Ritz-Sedlacek V, Jacob U, Gerardy-Schahn R (2004) Structure and function of vertebrate CMP-sialic acid synthetases. Glycobiology 14:43R–51R
17. Dommerholt J, Schmidt S, Temming R, Hendriks LJ, Rutjes FP, van Hest JC, Lefeber DJ, Friedl P, van Delft FL (2010) Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. Angew Chem Int Ed Engl 49:9422–9425
18. Verdurmen WP, Wallbrecher R, Schmidt S, Eilander J, Bovee-Geurts P, Fanghanel S, Burck J, Wadhwani P, Ulrich AS, Brock R (2013) Cell surface clustering of heparan sulfate proteoglycans by amphipathic cell-penetrating peptides does not contribute to uptake. J Control Release 170:83–91