Journal of Controlled Release 180 (2014) 81–90

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Glycosaminoglycans in the cellular uptake of drug delivery vectors – Bystanders or active players?

Marco E. Favretto [1], Rike Wallbrecher [1], Samuel Schmidt, Romy van de Putte, Roland Brock *

Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, Geert Grooteplein 28, 6525 GA Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 17 December 2013
Accepted 9 February 2014
Available online 16 February 2014

Keywords:
Cell-penetrating peptides
Drug delivery
Endocytosis
Glycocalyx
Syndecan
Virus

ABSTRACT

The implementation of efficient strategies for cellular delivery is the most significant hurdle in the development of oligonucleotide and protein-based nanomedicines. Unlike small molecule drugs that enter cells by virtue of hydrophobicity or by being substrates of transporters, these macromolecules lack the capacity to cross the plasma membrane in a non-disruptive way, therefore requiring the combination with carriers that mediate entry. Remarkably, for the major part, these carriers lack distinct structural features except for a high density of positive charge. Uptake has been attributed to the ability to engage in electrostatic interactions with the lipid bilayer and negatively charged glycosaminoglycans (GAGs) of the cellular glycocalyx. However, conflicting evidence has been obtained to which degree the interaction with GAGs contributes to uptake and the molecular mechanisms involved in uptake. Also, it is not clear to which extent the same molecular mechanisms apply for the different types of cationic delivery vectors. Here, we review the available data for cationic delivery vectors, including lipoplexes, polyplexes and cell-penetrating peptides (CPPs). We show that in spite of their different molecular size and degree of positive charge, all types of vectors share major characteristics with respect to the suggested role of GAGs in uptake. Moreover, by a comparison with the role of heparan sulfates in viral uptake we propose new avenues in the search for molecular mechanisms that trigger uptake of drug delivery vehicles and discuss how these insights may translate into new design principles for nanomedicines.

Contents

1. Introduction

The lipid bilayer of the plasma membrane forms the boundary between the inside and the outside of a cell. However, molecules approaching a cell will first encounter a layer of oligosaccharides that

* Corresponding author: Tel.: +31 24 3666213; fax: +31 24 3616413.
E-mail address: r.brock@ncmls.ru.nl (R. Brock).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.jconrel.2014.02.011
0168-3659/

can take the form of a dense glycocalyx as thick as 2–4.5 μm for capillary endothelial cells [1]. This glycocalyx is formed by the N- and O-linked glycosylation of the extracellular domains of transmembrane and membrane-associated proteins in which the terminal structures can contain the negatively charged sialic acid and sulfated sugars [2,3]. In particular, the proteoglycans carry large O-linked oligosaccharides consisting of highly negatively charged repeating disaccharide units, the glycosaminoglycans (GAGs), such as the heparan sulfates (HS).

The glycocalyx fulfils numerous functions ranging from an impact on the elastic behavior of the plasma membrane and the cell in its tissue context to the modulation of the local ionic environment through binding of water and counter ions [4,5]. Next to these non-specific functions, the glycocalyx also provides binding sites for growth factors and thus influences the cellular microenvironment with respect to cell proliferation and differentiation. Basic fibroblast growth factor 2 may be considered the paradigmatic example for a role of the glycocalyx in growth factor function, as activity of this growth factor towards its receptor depends on the simultaneous binding to HS [6].

Next to growth factors, the glycocalyx has also been associated with the cellular binding and uptake of several viruses. In this context, the question has been addressed to which extent binding of these pathogens to the glycocalyx by itself triggers intracellular signaling leading to uptake or whether binding to the glycocalyx promotes uptake by inducing proximity to receptors involved in uptake [7–10].

Also for polycationic nanoparticles and cationic cell-penetrating peptides (CPPs), binding to GAGs has been linked to uptake [11]. Nanoparticulate delivery systems share with viruses the size and capacity to engage in multivalent interactions. Yet, they lack specific domains for cell surface receptors that control cell tropism and triggering of uptake for most viruses. A comparison of viruses and nanoparticles should therefore help to reveal functions that GAGs can exert by themselves and point towards molecular mechanisms that so far have been overlooked in the uptake of nanoparticles.

CPPs, which are peptides of about 8 to 30 amino acids in size [12], do not interact with specific receptors. The current mechanistic framework for induction of uptake comprises cross-linking of heparan sulfates which then induces activation of small GTPases that lead to actin reorganization followed by endocytosis [13]. In spite of its appeal, evidence to support this model is still fragmentary and a number of observations do not fit into this concept. Moreover, one should note that whenever a reduction of uptake by removal of heparan sulfates has been reported, this reduction varied between only 20 and 80% [14,15].

The current state of knowledge therefore warrants a critical evaluation of the available data and concepts of the role of GAGs. In fact, the available data supports three different hypotheses for the role of GAGs in uptake: (i) GAGs cluster upon ligand binding which then drives uptake, (ii) GAGs bind the ligand and then cocluster with (a) receptor(s) and (iii) GAGs are involved as coreceptors of unknown receptor(s) binding the ligand at the surface and bringing it in close proximity to the receptor.

In this article, we review the latest findings in the light of any of these three hypotheses. Following an overview of the structure and function of GAGs and associated proteins, we will review the evidence that links GAG binding to uptake of CPPs and polyplexes. We will then provide a synthesis of the individual concepts to identify commonalities but also aim to stimulate a cross-validation by motivating experiments that test concepts across types of vectors. We will also discuss the GAG dependence of virus uptake as it may stimulate new avenues for research on the uptake mechanisms of the drug delivery vectors.

A better understanding of the role of GAGs in uptake will be instrumental in the rational development of delivery vectors with higher efficacy and also a preference for certain cell types. Also, a clear understanding of the dependence of GAG binding and proteins involved in delivery may enable a prediction of the tissue tropism of delivery vectors. Based on such insight decisions on the incorporation of further targeting modalities, for example directed against certain cell surface receptors, can be made.

1.1. Heparan sulfate proteoglycans — molecular structure and roles in cellular signaling and initiation of endocytosis

Heparan sulfate proteoglycans (HSPGs) comprise a diverse group of proteins that contain at least one covalently bound heparan sulfate (HS) glycosaminoglycan (GAG) chain and can carry chondroitin sulfate (CS) and dermatan sulfate (DS) (Fig. 1) [16]. GAGs can consist of more than 150 repeating disaccharide units and the number of GAG chains attached to a protein core (10 kDa–500 kDa) varies from one to more than 100, depending on the type of HSPG [17,18]. The typical density of HS proteoglycans on the cell surface as measured in various cell culture systems are in the range of 10^5 to 10^6 molecules per cell [19]. The large number of further structural modifications, such as sulfation and acetylation, provides the possibility to create a large structural diversity and the basis for generating specific domain structures which can be utilized for biological recognition of proteins [18].

With respect to a potential role as cellular attachment sites for CPPs, polyplexes and viruses, there are two major groups of cell-associated HS proteoglycans which differ from each other in their core protein: the glypicans (GPCs) and the syndecans (SDCs) (Fig. 1). In contrast to syndecans, glypicans do not possess any intracellular domain and by virtue of their membrane-proximal positioning of GAG chains, they are considered to work exclusively as co-receptors, as for fibroblast growth factor-2 (FGF-2), and as 'fine tuners' of cell signaling [27,28]. SDCs are transmembrane proteins and in human and other mammals four different SDCs have been described that differ in the nature of coupled oligosaccharides, structure of intracellular signaling domains and tissue distribution. In general, SDCs carry three to five HS chains and SDC-1 and -3 may also contain some CS/DS chains [29]. While SDC-4 is ubiquitously expressed, the other SDCs are only expressed on certain cell types: SDC-1 on epithelial and plasma cells and SDC-2 on endothelial cells and fibroblasts [29]. SDCs are involved in various cellular processes, such as cell adhesion and migration [29]. They have also been broadly associated with endocytosis of nanoparticulate systems and CPPs as recently reviewed by Belting [11]. Given their temporally and spatially controlled expression patterns and the phenotypes of knock-downs, it is clear that SDCs exert important functions in development and tissue organization. However, it is difficult to define whether the SDCs exert these functions in isolation or if they act as co-receptors through a local enrichment of growth factors via their GAG chains and signaling mediated by their C-terminal intracellular domain. This domain can serve as a scaffold for the organization and molecular interaction with signal transduction proteins that have an impact on cytoskeletal organization and initiation of endocytosis (Fig. 2) [11,29,30]. A prominent feature of the intracellular domains of all SDCs is the presence of a C-terminal PDZ motif that binds to PDZ domain containing proteins [31]. Also for the other intracellular and transmembrane domains a variety of interactions has been reported [30]. Nevertheless, many of the reported interactions may be indirect. The most obvious link to autonomous signaling so far is the activation of protein kinase C by SDC-4 [32–34] leading to the activation of the small GTPases Rac, RhoA and Cdc42 which are all involved in actin reorganization and thereby can induce the internalization of this HSPG. However, input from integrins may be required [35,36]. Given that SDCs functionally synergize with other cell surface receptors, as for example integrins, the extent to which syndecans can be understood as true autonomous receptors capable of driving actin reorganization and uptake awaits further clarification.

1.2. Heparan sulfate proteoglycans in the uptake of CPPs

1.2.1. Structural determinants for HS binding

In spite of the evidence linking GAGs to uptake these molecules have never been considered as CPP receptors in the classical sense. To a large part, this notion may still be attributed to observations that were initially described as key characteristics of CPP uptake such as energy and chirality independence, and absence of saturation [48,49]. However, in the

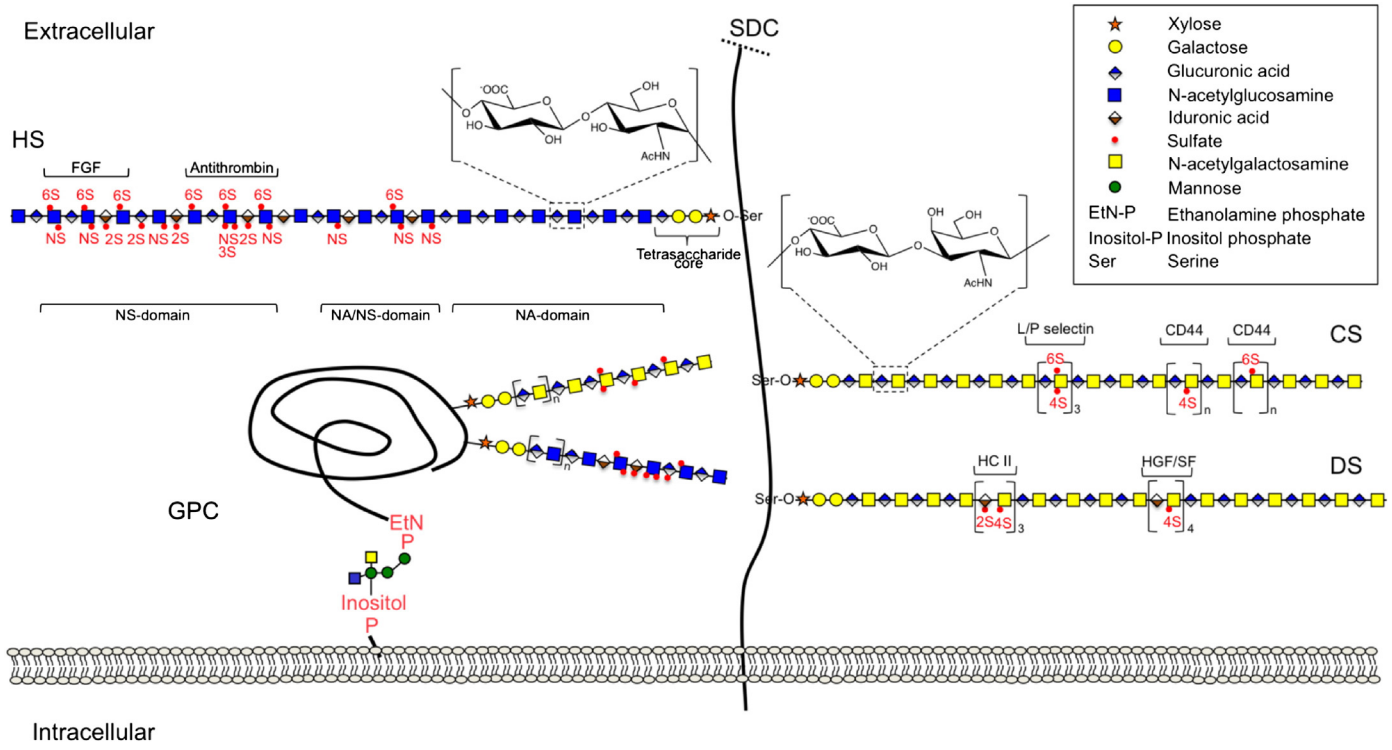

Fig. 1. Schematic representation of the disaccharide composition of different GAGs (heparan sulfate (HS), chondroitin sulfate (CS) and dermatan sulfate (DS)) linked to syndecan (SDC) and glypican (GPC). These GAGs comprise a uniform tetrasaccharide core (xylose–galactose–galactose–glucoronic acid), covalently bound to a Ser/Thr residue of the core protein [20]. HS comprises an N-acetyl-glucosamine (GlcNAc) and a uronic acid (UA) residue and undergoes modification reactions via epimerization of some glucoronic acid units to iduronic acid. Furthermore, sulfation can occur at different positions within the polymer, including N-deacetylation and N- and O-sulfation of the N-acetylglucosamine units (GlcNAc) [21]. These modifications generate various domain structures, e.g. for binding of FGF [22,23]. CS and DS consist of repeating disaccharide units of N-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA) [24]. Also CS and DS can be modified by partial epimerization, sulfation and N-deacetylation/N-sulfation to form distinct recognition epitopes for potential binding partners [25,26].

meantime a large body of evidence has been compiled that refutes each of these initial concepts. Importantly, GAGs have been associated with energy-dependent endocytosis (see below). Also the fact that oligoarginines bind to heparan sulfates with binding constants in the upper nanomolar range [50–52], suggests that GAGs may in fact be considered as CPP receptors raising the question whether specific structural determinants for binding and induction of uptake exist.

For the interaction of cationic CPPs with GAGs, the number of positive charges and the number of arginines, in particular, were shown to be critical [15]. Studies conducted on the interaction of the Tat peptide with heavily sulfated saccharides (heparin, heparan sulfates and chondroitin sulfate) demonstrated that the number of binding sites per unit of molecular weight differed for the different types of GAGs and correlated roughly with the degree of sulfation [53]. Electrostatic forces between positively charged amino acid residues and negatively charged sulfates drive the peptide–sugar interaction, as demonstrated by a detailed analysis of the thermodynamics of binding of nonaarginine [51]. The dependence of the stoichiometry of binding on the degree of sulfation suggested charge neutralization as a molecular principle for binding; moreover, the authors hypothesized that nonaargines can cluster HS. Along with electrostatic forces, hydrogen-bonding and/or hydrophobic interactions were also reported to be involved in the interaction: for two penetratin variants, differing in the numbers of arginines and lysines, hydrophobic interactions were critical in the binding of the arginine-containing analog with heparin, while binding of the lysine-containing variant was mainly mediated by electrostatic interactions [54]. Moreover, for a series of penetratin analogs that differed in the number of tryptophans, there was a strong positive correlation between the number of tryptophan residues and affinity [55]. GAG binding led to the formation of aggregates in a tryptophan content-dependent manner and the degree of binding correlated with uptake.

Remarkably, none of the studies investigating the interaction of CPPs with GAGs addressed the requirement for a specific sequence motif. In comparison, in 1989, Cardin and Weintraub performed a screening of 21 HS-binding molecules and found that they all contained [XBBXBX] as a consensus sequence, with B corresponding to a basic amino acid and X corresponding to an arbitrary (or hydrophobic) amino acid [56]. For a CPP derived from human lactoferrin [52] this motif is present but not essential [57]. In fact, due to the high density of positively charged residues most cationic CPPs will more or less conform to this motif.

1.2.2. The role of HS in CPP uptake

The reduction in uptake upon enzymatic removal of heparan sulfate chains, and the poor internalization of CPPs in mutant Chinese hamster ovary (CHO) cell lines lacking either all GAGs or only HS [14,15,52,57–59] support the notion that proteoglycans play a central role in the uptake of CPPs. However, it cannot be excluded that the GAG-deficient cell lines might also be defective in functions other than GAG synthesis which might affect uptake. Furthermore, the GAG-deficiency may have induced compensatory adaptations that may affect the results.

Competition with dextran sulfates or heparin has also been employed to address the importance of the capacity of CPPs to interact with cell-surface heparan sulfates for uptake. Coincubation of cells with dextran sulfate or heparin decreased uptake efficiency [60]. However, this was also the case for Jurkat cells that are poor in HS [61]. Therefore, these experiments rather show that CPP possess the capacity to bind to HS and that this binding reduces their uptake activity but do not reveal whether HS are essential for uptake.

Evidence has been presented that the role of GAGs is concentration dependent. Jiao and coworkers showed for penetratin and arginine-rich peptides that at low concentrations (1 μM), GAGs are by-passed, whereas

at higher peptide concentrations, GAG-dependent endocytosis occurs [62].

In spite of this evidence in favor of a role of GAGs in uptake, there are also studies describing an inhibitory effect of GAGs [63] or sialic acid [64] in peptide uptake indicating that sequestering of peptides by GAGs or sialic acid may impede uptake. At this point, the basis for the reported discrepancies is not clear.

1.2.3. The role of CPP-induced GAG clustering for uptake

CPPs possess the capacity to trigger endocytosis and clustering of HS has been associated with this activity [65,66]. Even though the exact mechanism of HS clustering is still not understood, the general idea is that charge neutralization between the cationic peptides and the anionic GAGs leads to the formation of neutral nanoparticulate complexes which are internalized [15,67–69]. However, controversy exists to which degree clustering is directly related to uptake. For variants of a CPP derived from the human protein lactoferrin [52], there was a strong negative correlation between the stoichiometry of binding and uptake efficiency [57] (Fig. 3a). However, uptake did not correlate with the capacity of these peptides to cluster HS in solution. Variants of penetratin containing lysines and arginines engage HS and internalization is correlated with the arginine content and the clustering efficiency [54] (Fig. 3b). However, the arginine-containing variant is also internalized more efficiently in the absence of HS indicating that the ultimate trigger for uptake is not directly related to HS, an observation that is consistent with our observation that L- and D-variants of arginine-rich peptides bind to HS with similar affinity, yet the latter are less efficient in triggering uptake [50] (Fig. 3c).

A PEGylated version of an arginine-tryptophan peptide that failed to cluster HS due to the steric hindrance imposed by the PEG chain entered cells via endocytosis also questioning the role of HS clustering in the induction of endocytosis [67]. Our group recently showed that for analogs of the amphiphilic CPP TP10 there was no correlation between GAG clustering and internalization [61] (Fig. 3d).

A more differentiated view was proposed by Rullo and coworkers who suggested that the formation of tight and stable complexes, as observed for penetratin with isolated components in a cell-free system, can be translated into endocytic import, while loosely bound peptides such as Tat are likely to enter the cell via direct translocation [70]. In spite of its appeal, this idea is difficult to reconcile with the observation for the PEGylated CPP and the fact that D-peptide analogs are less effective in inducing endocytosis in spite of having the same affinity for HS as their L-counterparts [50]. Since the heparan sulfates themselves do not show a chiral discrimination of CPPs, additional factors, as for example receptors, must be involved in the triggering of uptake as indicated above.

1.2.4. Signaling processes involved in peptide internalization

Except for those situations in which CPPs were shown to passively enter cells by direct crossing of the plasma membrane [62,71,72], the interaction of the CPP with the cell initiates processes that lead to uptake either via endocytosis [65,66] or by activation of acid sphingomyelinase [73].

Concepts on how this interaction is coupled to the activation of endocytosis are vague. Two lines of evidence are being followed. One line addresses the potential of the CPPs to autonomously induce

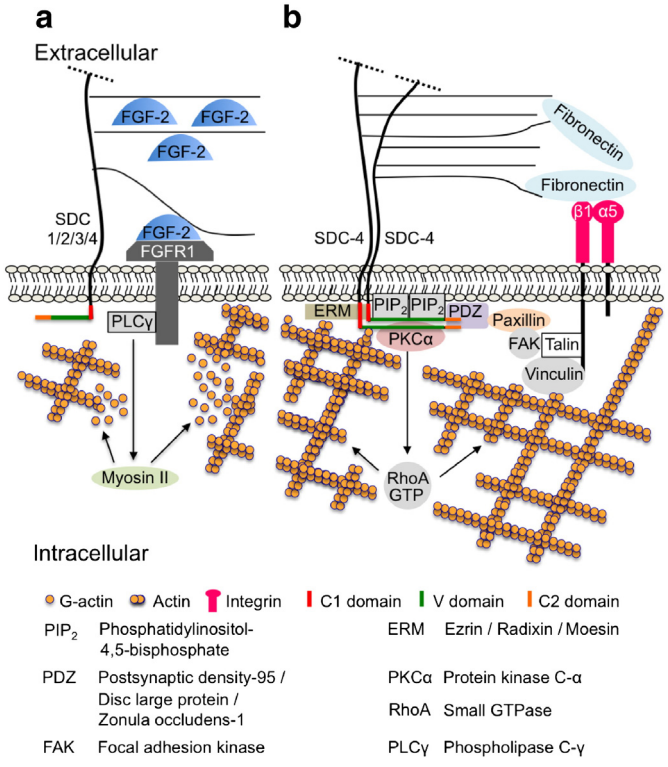

Fig. 2. Syndecans as coreceptors in signaling. a) Link of HS chains on HSPGs and cytoskeletal reorganization as exemplified for FGF-2-dependent signaling by syndecans [6]. The HS chains of syndecan-1–4 HSPGs play an essential role in fibroblast growth factor (FGF) signaling via direct interaction of the GAGs with FGF and its receptor FGFR-1 [6,37–39]. This interaction facilitates the formation of a ternary complex on the cell surface and consequent PLCγ and myosin II-mediated actin reorganization [40–43]. b) The presence of the extracellular matrix component fibronectin facilitates the coreceptor function of SDC-4 in fibronectin-induced integrin signaling. The cytoplasmic part of SDCs is divided into three domains: the conserved domains 1 and 2 (C1 and C2) and a variable (V) domain [44]. These domains contain recognition sites in C1, as for the ERM protein family, tubulin and cortactin. The C2 domain comprises a binding site for PDZ domain-containing proteins, e.g. syntenin, synectin, synbindin, and calcium/calmodulin-dependent serine protein kinase. The function of the V domain in SDC-1, -2 and -3 is largely unknown, whereas for SDC-4 a binding site for phosphatidylinositol-4,5-bisphosphate (PIP2) within the V domain was found to be responsible for binding of SDC-4 to PI-4,5-bisphosphate (PIP2) and protein kinase C-α (PKCα). This interaction activates PKCα, which in turn phosphorylates cytoplasmic proteins involved in focal adhesion formation and thereby links SDC-4 to the actin cytoskeleton and consequent reorganization [45–47].

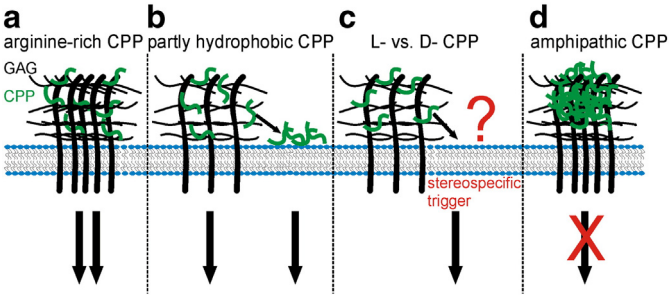

Fig. 3. Proposed models for the internalization of CPPs. a) The internalization of arginine-rich CPPs occurs in a stoichiometry-dependent way pointing towards an autonomous role of GAGs for the internalization of CPPs [57]. b) For CPPs containing the hydrophobic amino acid tryptophan it was shown that, next to interactions with GAGs, direct binding to the membrane is promoting uptake [54]. c) D-CPPs are internalized less efficiently than the L-counterpart despite of having the same HS affinity. This observation suggests the involvement of a stereospecific, yet unknown, trigger [50]. d) Amphipathic peptides were shown to form clusters with GAGs, which are, probably due to their size, not internalized [61].

membrane curvature and actin reorganization [74–76]. However, so far this research has been limited to cell-free model systems and the validation of the physiological relevance is hampered by the difficulty to study these mechanisms in cells in isolation. To this point, none of these models has incorporated GAGs as potential binding sites.

A second line of evidence links the clustering of heparan sulfates to the induction of actin rearrangement leading to endocytosis. This model is based on the observation that exposure of cells to CPPs leads to lamellipodia formation in a Rac-dependent manner [13]. However, while this latter publication demonstrated the abolishment of lamellipodia formation in the presence of dominant negative Rac1, it lacks the final evidence which would be inhibition of CPP uptake. Imamura and coworkers performed elegant single particle tracking experiments with Tat-conjugated quantum dots. Interestingly, only exposure of cells to multivalent, but not to bivalent quantum dots led to Rac1-dependent local actin reorganization and uptake [77].

GAGs, and syndecans were placed into this model by showing that the overexpression of syndecan-2 [15] or -4 [78] resulted in increased peptide uptake. The link to syndecan-4 was further supported by demonstrating that the protein kinase C inhibitor Gö6976 also reduced uptake.

In contrast, the observation that preincubation with exogenous dextran sulfate can restore the uptake of short cationic peptides in GAG-deficient cells suggests that induction of uptake is independent of the core protein [79]. This latter evidence also raises the possibility that heparan sulfate proteoglycans only act as auxiliary receptors. This concept is exemplified by the interplay of HS and the low-density lipoprotein receptor-related protein (LRP) for the uptake of lipoproteins [80]. Here, HS serve as attachment sites of remnant lipoproteins which are then passed to the LRP which acts the receptor mediating uptake. Also, for the full length HIV-Tat protein on neuronal cells, the interaction of the Tat core domain with LRP was linked to endocytosis while the initial cellular attachment occurred through interaction of the Tat basic domain with HS [81].

1.3. Heparan sulfate proteoglycans in the uptake of nanoparticulate systems

Glycosaminoglycans have been recognized as a determinant also for the internalization and intracellular activity of nano- and microparticulate drug delivery systems. Since most delivery vectors are polycationic, the charge-driven interaction of cationic particulate systems with heparan sulfates has been considered common sense. However, the recent observation that in serum, polyplexes assume a negative zeta-potential and that uptake may occur via scavenger receptors also stresses the importance for further research in this field [82,83]. Also, the question arises to which extent differences in the interpretation of results may be the consequence of the use of serum-free versus serum-containing media. While for CPPs the presence of serum only reduces uptake efficiency [84] for polyplexes, the impact of serum and as a consequence change of zeta potential may lead to qualitatively different uptake mechanisms. Furthermore, for polyplexes, heparan sulfates may also play a role in the decomplexation and intracellular fate of the complexes. The intracellular trafficking of polyplexes is an area of research on its own. Therefore, here we will exclusively focus on uptake.

1.3.1. The role of HS in the cell attachment and uptake of poly- and lipoplexes

Evidence of the involvement of GAGs in the uptake of polyplexes was reported first during the late 1990s. Mislick suggested that GAGs can act as receptors for gene delivery complexes both, *in vitro* and *in vivo* and that this interaction is responsible for the cellular entry of the complexes [85]. Moreover, these pioneering studies concluded that electrostatic interactions are required for triggering internalization of the complexes. This hypothesis is also supported by more recent studies, demonstrating that positively charged DNA carriers with very different molecular structure, including PEI [86–88], cyclodextrins [89], poly(amido amines) [90] and poly(glycoamidoamines) [91] engage GAGs before being internalized (Fig. 4a).

Most studies have focused on the impact of heparan sulfates using GAG-deficient cell lines or enzymatic cleavage. While this may provide general insights into the role of cell-bound polyanions for uptake, further insights can be obtained by a discrimination of specific structural determinants, as exemplified by the analysis of the cellular uptake of poly(glycoamidoamines) [91]. Upon desulfation of GAGs, which reduces the negative charge on these molecules, only a modest reduction in cellular uptake of PEI polyplexes but a dramatic reduction of internalization of poly(glycoamidoamines) was observed. Since desulfation had little effect on uptake of PEI, these authors suggested that other GAG such as CS and DS can provide the required functionalities. One may comment, however, that CS and DS should also be desulfated as a consequence of the treatment, questioning the validity of this interpretation. An analysis of the interaction of the polyplexes with various GAGs suggested that next to electrostatic interactions, other interaction forces are also involved.

By studying different CHO cell clones, Thompson and coworkers showed that a high density of HS promoted uptake [87]. However, one clone also showed high binding but low internalization, suggesting that uptake is a function of the relative abundance of different GAG-carrying proteins rather than the overall HSPG level. This study also demonstrated that the use of different clones of one cell line may introduce substantial experimental variability between laboratories.

Decomplexation of polyplexes by GAGs may be a confounding factor in analyzing the role of cellular GAGs in uptake [92]. DNA can be released prematurely from cationic poly- or lipoplexes through interaction with anionic HS and this interaction strongly depends on the nature of the oligoplexes and the kind of GAG expressed on the cell surface [90] (Fig. 4b) [91]. The fact that HS removal can even increase uptake of PAMAM polyplexes [93] can be explained by an increased maintenance of polyplex integrity on the cell surface.

In spite of this strong evidence supporting a role of HS in uptake, it is important to keep in mind that upon HS removal uptake is never fully

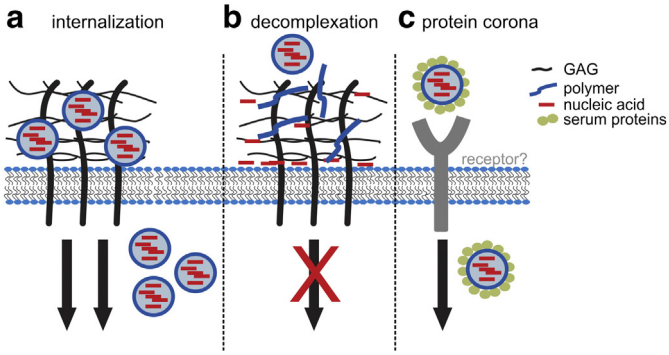

Fig. 4. Suggested models for the uptake of nanoparticulate complexes. a) GAGs are inducing the internalization of nanoparticulate complexes [86]. b) Binding to GAGs leads to the decomplexation preventing uptake [92]. c) Serum proteins may bind to the cationic polymers which are encapsulating the nucleic acids. The internalization of these particles with the protein corona may occur through the scavenger receptor [83].

eliminated but only reduced by amounts similar to the ones described for CPPs. Nevertheless, to this point most data supports a positive role of HS in internalization raising the question whether HS do so by acting as mere attachments sites, or whether binding of HS triggers uptake.

1.3.2. Endocytosis of cationic nanoparticulate systems

Following binding to the cell surface, the consensus is that gene delivery systems enter the cells by endocytosis or phagocytosis. It has been demonstrated that internalization of polyplexes has an impact on growth factor signaling, providing evidence that similar to CPPs polyplexes induce endocytosis and do not just "piggy back" on constitutive membrane turnover [94]. The absence of any specific structural requirement of polyplexes except for cationicity for uptake, leaves the induction of HS clustering as the most obvious molecular mechanism triggering uptake. So the questions are, whether clustering is indeed sufficient as a mechanistic concept and what are the factors that trigger internalization of DNA complexes.

Mounkes [95] observed that lipoplexes were internalized to a high extent by cells (over)expressing SDC-1; furthermore, heparinase exposure decreased transfection also *in vivo*. SDC-1 was also shown to be important for the internalization of polyplexes [88,96,97]. Nevertheless, these studies proposed different mechanisms to explain the role of syndecans in internalization.

Kopatz and coworkers [96] proposed a detailed sequence of events that were defined as HS-dependent phagocytosis. According to their model, after initial binding, a gradual electrostatic engulfment of the plasma membrane around the particle occurs and this zippering is sustained by the clustering of SDC-1 in cholesterol-rich rafts. This clustering in turn triggers PKC activity and causes the linking of actin binding to the cytoplasmic tail of the syndecan. The resulting tension then pulls the particle into the cells. However, this model seems to apply only to large particles (200–500 nm), as well as bacteria and viruses.

An expansion of this model was proposed by Paris and coworkers [97]. These researchers showed that SDC-1 is involved in the recognition and binding of the complexes, while SDC-2 acts as an accessory molecule. Upon binding of the polyplexes, clusters of SDC-1 and -2 are formed. However, only clustering of SDC-1 seems to be involved in the internalization of the complexes as 1 h after incubation, mostly SDC-1/polyplex complexes were internalized and localized in the proximity of the nucleus, while SDC-2 clusters were still located at the plasma membrane in association with polyplexes. Interestingly, upon co-expression of both syndecans, SDC-2 exerted a dominant negative effect on transfection since in this case syndecan/polyplexes remained at the cell surface even after 6 h.

By studying the events that occur prior to endocytosis, Rehman and coworkers showed that polyplexes and lipoplexes attach via, both, SDC-1 and -2, leading to syndecan clustering and induction of actin polymerization, which then causes retrograde transport of the syndecan–ligand complex along filopodia [88]. Inhibition of filopodia movement highly reduced transfection efficiency. However, it was not shown whether this was only due to a lack of transport of complexes to the cell bodies or whether endocytosis of complexes was also compromised.

With respect to other proteins involved in uptake, it was demonstrated that dynamin is required for the internalization of proteoglycan-binding ligands through a caveolin- and clathrin independent but flotillin and dynamin-dependent mechanism [86]; along with dynamin, the presence of flotillin-1, a component of lipid rafts, promoted the internalization of polyplexes in agreement with results obtained for poly(amido amine) polyplexes.

In spite of this evidence linking SDCs to uptake, as for CPPs also for polyplexes a scarcity of knowledge exists in how heparan sulfate engagement is coupled to the induction of endocytosis. Moreover, the recent demonstration of an involvement of scavenger receptors in the uptake of polyplexes demonstrates that there is room for radically new concepts [83]. In the presence of serum, an inversion of the zeta-potential at least for polyplexes consisting of CPP and oligonucleotides occurs likely due to formation of a protein corona (Fig. 4c) [82,83,98,99]. These observations question the general view of an interaction driven primarily by electrostatics.

1.4. Heparan sulfates in viral infections

Even though the entry of viruses is typically associated with more classical receptor ligand interactions, syndecans have also been associated with viral uptake (Fig. 5). Heparan sulfates have been implicated as attachment molecules in the infection of herpes simplex virus (HSV), [100] and a mechanism of HS-mediated "surfing" along filopodia, similar to the one described for polyplexes, has been observed [101]. For HSV-1, downregulation of the syndecans or blockage by antibodies resulted in a decreased infection and less entry into the cells. Furthermore, upon infection, expression levels of syndecan-1 and syndecan-2 are increased [102].

As first shown for HeLa cells [103], and lately for physiological target cells lacking CD4, such as macrophages [104] and dendritic cells [105], attachment of HIV to cells is mediated by syndecans. On dendritic cells, HIV seems to engage syndecan-3 [105] via a polybasic stretch in glycoprotein gp120, the classic ligand on the HIV surface [106]. However, this interaction does not lead to uptake. Instead, on dendritic cells the virus forms a reservoir for infection of CD4-positive T-helper cells [105].

For human papillomavirus (HPV), that infect terminally differentiated epithelial cells, SDC-1 serves as the initial receptor through binding to lysine residues in the capsid proteins. This binding induces conformational changes in the capsid proteins required for cell entry [107]. However, these conformational changes do not require HS [7]. Also here, the transport of virions along actin-rich protrusions bears remarkable similarity to the one shown for PEI particles [88]. Evidence suggests that a further, yet unknown receptor, is associated with viral entry and subsequent infection [7]. Surviladze and coworkers proposed a model

in which uptake is induced by growth factors associated with soluble HS bound to the virus particles [108].

HS have also been considered as attachment factors in the infection of Dengue virus, through binding of the envelope glycoprotein E. Glycoprotein E has several binding sites for HS that promote both, attachment and cell entry [109]. For a particular dengue virus strain syndecan-2 was identified as a receptor [110]. Bound particles laterally diffuse in the plasma membrane until they are captured by preformed clathrin-coated pits which then serve as the sites of viral entry [8]. Nevertheless, binding of HS, although necessary, may not be the only factor required for internalization of the viral particle. Intracellular signaling mediated by G protein-coupled receptor kinase 2 seems to be implicated in the triggering of endocytosis [9].

Syndecans are also involved in the first step of hepatitis E virus (HEV) infection. In particular, SDC-1 mediates viral attachment to host cells, as its removal dramatically decreases HEV binding [111]. Uptake of HEV was shown to occur via clathrin-dependent endocytosis [112]. Researchers posit that a receptor for HEV has to exist, indicating that SDC-1 acts as an accessory molecule or as a co-receptor.

For human T-cell leukemia virus type I (HTLV-1) syndecans bearing short-chain heparan sulfates contribute to uptake. This publication stresses the requirement for the short-chain nature of the HS chains leading to the concept that these short-chain HS position the virus close to the plasma membrane [10]. This activity acts in concert with the glucose transporter 1 and neuropilin-1 for induction of uptake.

Overall, for viral entry HS act as accessory molecules and uptake has been linked to the induction of down-stream processes linked to the primary receptor molecules rather than the proteoglycans.

1.5. Bystanders or active players?

With regard to our initial question — are GAGs bystanders or active players, the current state-of-knowledge reveals that this answer very much depends on the type of delivery system. As such, differences in the answer to this question may also rather be a consequence of the conceptional framework of the respective research area than true mechanistic differences.

Viral entry has historically been correlated to interactions with specific receptors, which eventually trigger a series of processes leading to internalization of the viral particles. In this scenario, GAGs were relegated to a secondary role as bystanders or coreceptors that support association of virus particles with the cell surface and at best contribute to uptake.

In contrast, studies carried out on CPPs and nanoparticulate systems have provided ample evidence that GAGs can act as autonomous receptors and therefore be the main actors in the internalization of such molecular entities. However, very clearly, in the complex context of the plasma membrane, it will never be possible to address the role of GAGs in isolation and to exclude an involvement of other receptor molecules. In fact, a considerable body of data supports the idea that the GAGs themselves are not sufficient to trigger the import of CPPs, questioning their role as the main active players. As one possibility, clustering of GAGs may induce co-clustering with a receptor that elicits import, probably via clustering-dependent activation of signaling processes (Fig. 5). Finally, binding to GAGs may bring the vector into the vicinity of a coreceptor. While this latter possibility may provide an explanation for the chirality dependence of endocytosis induction it is difficult to reconcile with the structural diversity of CPP sequences.

In the following section we will point out future lines of research that may resolve the question, whether for viruses and artificial delivery systems fundamental conceptional differences exist or whether major aspects in the uptake mechanisms of artificial vectors have been overlooked so far.

1.6. Future directions in the research of HS and uptake

So far, research on CPPs and polyplexes has been surprisingly separate. A potential reason may be that the CPP field had its origin in the area of transcription factors and membrane-active peptides, while polyplexes are a result of pharmaceutical polymer research. Very clearly, with the formation of CPP-based polyplexes, both areas overlap. Furthermore, also for free CPPs as shown above, in spite of the fact that polyplexes are much larger and polyvalent in nature, at present there seem to be more commonalities for CPPs and polyplexes than differences.

Very clearly, for CPPs, polyplexes, and viruses alike there is a strong link of HS engagement and uptake. However, conflicting data exist, and especially for CPPs and polyplexes the molecular events that link HS binding to the induction of uptake are sketchy. A direct visualization of the dynamics of vector, GAGs and candidate signaling proteins in living cells will be key to obtain such an understanding. However, the approaches used for GAG labeling so far are confronted with major shortcomings. For fusion to fluorescent proteins the structure of the

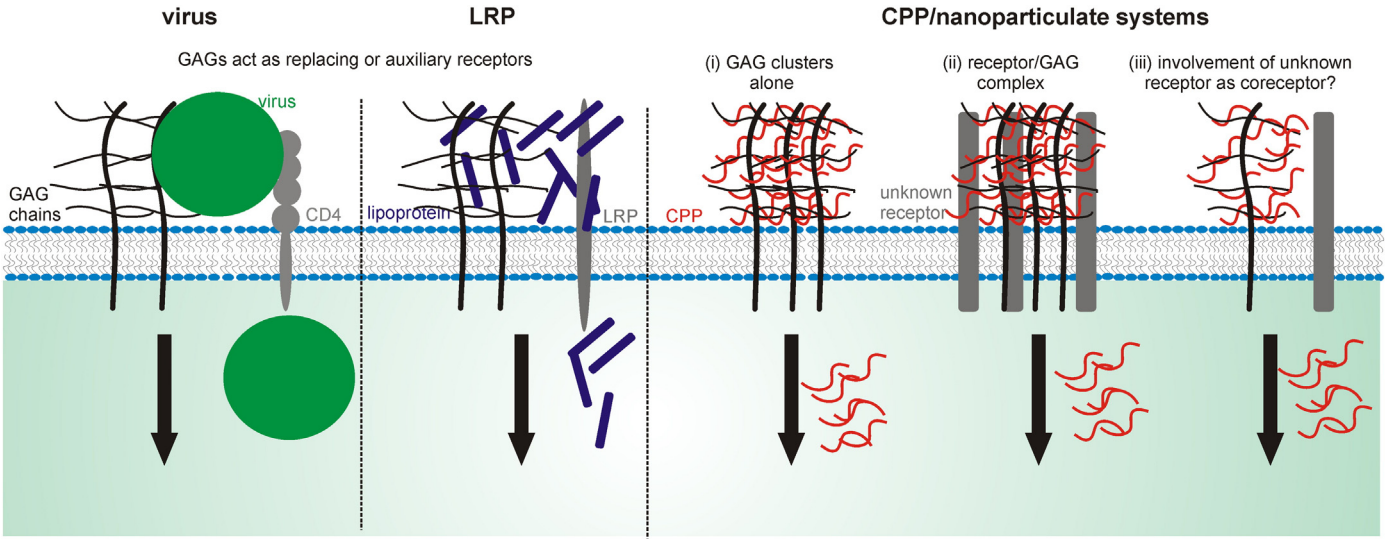

Fig. 5. Proposed models for the involvement of glycosaminoglycans in the uptake of viruses, proteins and CPP/nanoparticulate systems. For viruses and LDL receptor-related protein (LRP) HS bound to transmembrane proteins and in particular the syndecans were shown to act as coreceptors or auxiliary receptors. In contrast, for CPPs and other nanoparticulate complexes next to the current concept of GAG clustering we propose two more concepts in analogy to viruses and LRP: GAGs may act as a coreceptor for an unknown receptor or vice versa (as for LRP), or GAGs form clusters with the unknown receptor promoting uptake or complex formation of GAGs itself is already triggering the uptake.

syndecans and glypicans imposes major limitations. For the syndecans, a C-terminal fusion abrogates the functionality of the PDZ motif. Very interestingly, the publications that employed C-terminal GFP fusions of syndecan-1 did not include any mentioning of this point. Probably, the PDZ motif has little relevance as for C-terminal GFP fusions only after removal of intracellular domains delivery of PEI polyplexes was reduced [97]. Insertion of fluorescent proteins into the membrane proximal region of the extracellular domain [78] of syndecans and glypicans alike will change the positioning of oligosaccharides relative to the plasma membrane and as a consequence potentially impede with protein trafficking in the secretory pathway and protein–protein interactions at the cell surface.

Labeling with heparan sulfate-specific antibodies has been employed on living cells [113]. However, these reagents compete with the binding of vectors. For example, binding of the lactoferrin-derived CPP [52] could be inhibited completely by the HS-binding single chain antibody HS4C3 [114] (own unpublished results). Furthermore, secondary antibodies may artificially enhance crosslinking. Instead, metabolic labeling of sugars may present a highly powerful, yet little explored option for labeling of glycosaminoglycans [61,115,116].

As described for HIV, heparan sulfate-bound virus may even serve as a long-lived reservoir for infectious particles on the cell surface demonstrating that multivalent binding of heparan sulfates by nanoparticles does not necessarily lead to uptake. More research is therefore needed to understand the molecular basis for these differences. One possibility could be that viruses bind to heparan sulfates with a lower total positive charge density and that only this latter is associated with the triggering of delivery pathways of nanoparticles.

Given their role as accessory receptors in viral uptake, it could well be that the function of syndecans and glypicans in mediating uptake is context-specific, such as requiring the presence of further transmembrane proteins/receptors that ultimately trigger the uptake machinery (Fig. 5). Considering the fact that the local accumulation of many growth-factor receptors can trigger endocytosis, this concept would be fully compatible with the independence of uptake of a *specific* growth factor receptor that is a hallmark of uptake for CPP and polyplexes. The mechanisms triggering uptake of CPP and polyplexes would then be even more reminiscent of those triggering viral uptake or of LRP ligands [117].

Finally, more research is needed to elucidate the basis for the remarkable similarities in the uptake mechanisms of CPPs and nanoparticulate systems. Two possibilities can explain this circumstance: First, relevant differences may have been overlooked. Second, the observed coalescence of CPP–HS complexes into nanoparticulate structures on the cell surface may eliminate differences between both types of delivery vectors.

1.7. Potential implications for the design of nanomedicines

So far, the rationale for detailed molecular analyses in the cellular association and uptake of CPPs and polyplexes has been that if only uptake was understood in detail, then more efficient and less toxic delivery vehicles with ideal cell type specificity could also be generated.

For individual CPPs, significant effort has been invested into the analysis of structure–activity relationships of heparan sulfate binding and uptake. The question is to which extent this insight can benefit the further development of pharmaceutical polymers. For example are differences observed for penetratin and hLF variants [54,57] maintained if such a peptide is grafted onto a nanoparticle or does multivalency override these structural aspects? For cationic polymers and lipids further research is needed to elucidate whether the addition of a CPP benefits the delivery and which component controls uptake and intracellular trafficking.

Ultimately, the question remains, whether also with a GAG-centered approach, as currently followed for CPPs and polyplexes, an increased delivery efficiency in combination with cell type specificity and reduced toxicity can be reached. As summarized above, for proteins such as the LRP and its ligands and the Tat protein as well as for viruses, GAGs exert their physiological function in concert with other receptors through which cell-type tropism is attained. Except for those situations in which a targeting based on heparan sulfate density in the target organ confers sufficient specificity for therapeutic benefit, as is the case for siRNA targeting to the liver [118,119], research on CPPs and polyplexes could benefit from a consideration of GAGs as only auxiliary receptors. A deep investigation of the specific tissue distribution and relative abundance of syndecans and other heparan sulfate-carrying proteins may constitute a first step in this direction. Following such a strategy, it must be possible to rationally predict tissue preference of delivery systems and define the requirement for further targeting modalities.

Even more fundamental questions remain with respect to molecular determinants and mechanisms underlying cellular uptake of nanoparticulate systems. Very clearly, positive charge is a common denominator of delivery vectors. However, the observation that polyplexes assume a negative zeta-potential in the presence of serum adds another layer of complexity. The association of proteins with nanoparticles, leading to the so-called protein corona is an emerging area in nanoparticle research [98,99]. Still, a positive charge of the particle itself seems to be decisive for uptake [120], suggesting that the protein corona disassembles once a particle approaches the cell surface. Nevertheless, it is an intriguing concept that nanoparticles dynamically recruit factors contributing to their uptake, similar to what has been described for HPV [108]. In this case, research on nanomedicines needs to understand to which extent the protein corona is a function of the drug delivery vehicle and a consideration of the structure–activity-relationship of the drug delivery systems alone may be misleading. So far evidence of genome-wide gene expression analyses has indicated that nanoparticles enter without induction of gene expression changes [121] disfavoring an engagement of growth factor receptors. However, transferrin is also present in serum and, if absorbed to particles, could induce uptake without induction of growth factor dependent signaling. It remains to be shown, whether this dynamic self-organization of nanoparticles may be exploited to tailor organ tropism and targeting or whether ultimately, delivery strategies will focus on stealth strategies such as pegylation in combination with targeting moieties [122].

Acknowledgments

The authors acknowledge financial support from the Dutch Polymer Institute (M. E. F.), the BMBF Biotransporter initiative (R. W., 13N11454) and the Roche Postdoc program (S. S.). R. P. was supported by the Honors Programme of the Radboud University Medical Centre.

References

[1] S. Reitsma, D.W. Slaaf, H. Vink, M.A. van Zandvoort, M.G. oude Egbrink, The endothelial glycocalyx: composition, functions, and visualization, Pflugers Arch. 454 (2007) 345–359.
[2] P. Stanley, R.D. Cummings, Structures Common to Different Glycans, 2009.
[3] K.W. Moremen, M. Tiemeyer, A.V. Nairn, Vertebrate protein glycosylation: diversity, synthesis and function, Nat. Rev. Mol. Cell Biol. 13 (2012) 448–462.
[4] A.R. Pries, T.W. Secomb, P. Gaehtgens, The endothelial surface layer, Pflugers Arch. 440 (2000) 653–666.
[5] S. Weinbaum, J.M. Tarbell, E.R. Damiano, The structure and function of the endothelial glycocalyx layer, Annu. Rev. Biomed. Eng. 9 (2007) 121–167.
[6] A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder, D.M. Ornitz, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor, Cell 64 (1991) 841–848.
[7] P.M. Day, D.R. Lowy, J.T. Schiller, Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids, J. Virol. 82 (2008) 12565–12568.
[8] H.M. van der Schaar, M.J. Rust, C. Chen, H. van der Ende-Metselaar, J. Wilschut, X. Zhuang, J.M. Smit, Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells, PLoS Pathog. 4 (2008) e1000244.
[9] C. Le Sommer, N.J. Barrows, S.S. Bradrick, J.L. Pearson, M.A. Garcia-Blanco, G protein-coupled receptor kinase 2 promotes flaviviridae entry and replication, PLoS Negl. Trop. Dis. 6 (2012) e1820.
[10] A. Tanaka, A. Jinno-Oue, N. Shimizu, A. Hoque, T. Mori, S. Islam, Y. Nakatani, M. Shinagawa, H. Hoshino, Entry of human T-cell leukemia virus type 1 is augmented by heparin sulfate proteoglycans bearing short heparin-like structures, J. Virol. 86 (2012) 2959–2969.

[11] H.C. Christianson, M. Belting, Heparan sulfate proteoglycan as a cell-surface endocytosis receptor, Matrix Biol. (2013) http://dx.doi.org/10.1016/j.matbio.2013.10.004. [Epub ahead of print].
[12] F. Milletti, Cell-penetrating peptides: classes, origin, and current landscape, Drug Discov. Today 17 (2012) 850–860.
[13] S. Gerbal-Chaloin, C. Gondeau, G. Aldrian-Herrada, F. Heitz, C. Gauthier-Rouviere, G. Divita, First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling, Biol. Cell. 99 (2007) 223–238.
[14] J.P. Richard, K. Melikov, H. Brooks, P. Prevot, B. Lebleu, L.V. Chernomordik, Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors, J. Biol. Chem. 280 (2005) 15300–15306.
[15] I. Nakase, A. Tadokoro, N. Kawabata, T. Takeuchi, H. Katoh, K. Hiramoto, M. Negishi, M. Nomizu, Y. Sugiura, S. Futaki, Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis, Biochemistry 46 (2007) 492–501.
[16] J. Kreuger, D. Spillmann, J.P. Li, U. Lindahl, Interactions between heparan sulfate and proteins: the concept of specificity, J. Cell Biol. 174 (2006) 323–327.
[17] A.R. Poole, Proteoglycans in health and disease: structures and functions, Biochem. J. 236 (1986) 1–14.
[18] K. Prydz, K.T. Dalen, Synthesis and sorting of proteoglycans, J. Cell Sci. 113 (2000) 193–205.
[19] M. Bernfield, R. Kokenyesi, M. Kato, M.T. Hinkes, J. Spring, R.L. Gallo, E.J. Lose, Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans, Annu. Rev. Cell Biol. 8 (365–93) (1992) 365–393.
[20] K. Sugahara, H. Kitagawa, Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans, Curr. Opin. Struct. Biol. 10 (2000) 518–527.
[21] J.R. Bishop, M. Schuksz, J.D. Esko, Heparan sulphate proteoglycans fine-tune mammalian physiology, Nature 446 (2007) 1030–1037.
[22] S. Ashikari-Hada, H. Habuchi, Y. Kariya, N. Itoh, A.H. Reddi, K. Kimata, Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library, J. Biol. Chem. 279 (2004) 12346–12354.
[23] S. Ashikari-Hada, H. Habuchi, N. Sugaya, T. Kobayashi, K. Kimata, Specific inhibition of FGF-2 signaling with 2-O-sulfated octasaccharides of heparan sulfate, Glycobiology 19 (2009) 644–654.
[24] J.E. Silbert, G. Sugumaran, Biosynthesis of chondroitin/dermatan sulfate, IUBMB Life 54 (2002) 177–186.
[25] H. Kawashima, K. Atarashi, M. Hirose, J. Hirose, S. Yamada, K. Sugahara, M. Miyasaka, Oversulfated chondroitin/dermatan sulfates containing GlcAbeta1/IdoAalpha1-3GalNAc(4,6-O-disulfate) interact with L- and P-selectin and chemokines, J. Biol. Chem. 277 (2002) 12921–12930.
[26] J.M. Trowbridge, R.L. Gallo, Dermatan sulfate: new functions from an old glycosaminoglycan, Glycobiology 12 (2002) 117R–125R.
[27] A. Fico, F. Maina, R. Dono, Fine-tuning of cell signaling by glypicans, Cell. Mol. Life Sci. 68 (2011) 923–929.
[28] J. Gutierrez, E. Brandan, A novel mechanism of sequestering fibroblast growth factor 2 by glypican in lipid rafts, allowing skeletal muscle differentiation, Mol. Cell. Biol. 30 (2010) 1634–1649.
[29] J.R. Couchman, Transmembrane signaling proteoglycans, Annu. Rev. Cell Dev. Biol. 26 (2010) 89–114.
[30] K. Lambaerts, S.A. Wilcox-Adelman, P. Zimmermann, The signaling mechanisms of syndecan heparan sulfate proteoglycans, Curr. Opin. Cell Biol. 21 (2009) 662–669.
[31] M. Sheng, C. Sala, PDZ domains and the organization of supramolecular complexes, Annu. Rev. Neurosci. 24 (2001) 1–29.
[32] E. Keum, Y. Kim, J. Kim, S. Kwon, Y. Lim, I. Han, E.S. Oh, Syndecan-4 regulates localization, activity and stability of protein kinase C-alpha, Biochem. J. 378 (2004) 1007–1014.
[33] S.T. Lim, R.L. Longley, J.R. Couchman, A. Woods, Direct binding of syndecan-4 cytoplasmic domain to the catalytic domain of protein kinase C alpha (PKC alpha) increases focal adhesion localization of PKC alpha, J. Biol. Chem. 278 (2003) 13795–13802.
[34] E.S. Oh, A. Woods, J.R. Couchman, Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C, J. Biol. Chem. 272 (1997) 8133–8136.
[35] M.D. Bass, M.R. Morgan, M.J. Humphries, Integrins and syndecan-4 make distinct, but critical, contributions to adhesion contact formation, Soft Matter 3 (2007) 372–376.
[36] C.K. Thodeti, R. Albrechtsen, M. Grauslund, M. Asmar, C. Larsson, Y. Takada, A.M. Mercurio, J.R. Couchman, U.M. Wewer, ADAM12/syndecan-4 signaling promotes beta 1 integrin-dependent cell spreading through protein kinase Calpha and RhoA, J. Biol. Chem. 278 (2003) 9576–9584.
[37] D. Moscatelli, Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells, J. Cell Biol. 107 (1988) 753–759.
[38] D.A. Pye, R.R. Vives, J.E. Turnbull, P. Hyde, J.T. Gallagher, Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity, J. Biol. Chem. 273 (1998) 22936–22942.
[39] H. Rahmoune, H.L. Chen, J.T. Gallagher, P.S. Rudland, D.G. Fernig, Interaction of heparan sulfate from mammary cells with acidic fibroblast growth factor (FGF) and basic FGF. Regulation of the activity of basic FGF by high and low affinity binding sites in heparan sulfate, J. Biol. Chem. 273 (1998) 7303–7310.
[40] M. Mohammadi, S.K. Olsen, O.A. Ibrahimi, Structural basis for fibroblast growth factor receptor activation, Cytokine Growth Factor Rev. 16 (2005) 107–137.
[41] L. Haviv, D. Gillo, F. Backouche, A. Bernheim-Groswasser, A cytoskeletal demolition worker: myosin II acts as an actin depolymerization agent, J. Mol. Biol. 375 (2008) 325–330.
[42] I. Chandrasekar, J.E. Huettner, S.G. Turney, P.C. Bridgman, Myosin II regulates activity dependent compensatory endocytosis at central synapses, J. Neurosci. 33 (2013) 16131–16145.
[43] X. Sai, R.K. Ladher, FGF signaling regulates cytoskeletal remodeling during epithelial morphogenesis, Curr. Biol. 18 (2008) 976–981.
[44] M.R. Morgan, M.J. Humphries, M.D. Bass, Synergistic control of cell adhesion by integrins and syndecans, Nat. Rev. Mol. Cell Biol. 8 (2007) 957–969.
[45] A. Horowitz, M. Murakami, Y. Gao, M. Simons, Phosphatidylinositol-4,5-bisphosphate mediates the interaction of syndecan-4 with protein kinase C, Biochemistry 38 (1999) 15871–15877.
[46] J. Shin, W. Lee, D. Lee, B.K. Koo, I. Han, Y. Lim, A. Woods, J.R. Couchman, E.S. Oh, Solution structure of the dimeric cytoplasmic domain of syndecan-4, Biochemistry 40 (2001) 8471–8478.
[47] E. Tkachenko, J.M. Rhodes, M. Simons, Syndecans: new kids on the signaling block, Circ. Res. 96 (2005) 488–500.
[48] A. Prochiantz, Messenger proteins: homeoproteins, TAT and others, Curr. Opin. Biol. 12 (2000) 400–406.
[49] P.M. Fischer, E. Krausz, D.P. Lane, Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation, Bioconjug. Chem. 12 (2001) 825–841.
[50] W.P. Verdurmen, P.H. Bovee-Geurts, P. Wadhwani, A.S. Ulrich, M. Hallbrink, T.H. van Kuppevelt, R. Brock, Preferential uptake of L- versus D-amino acid cell-penetrating peptides in a cell type-dependent manner, Chem. Biol. 18 (2011) 1000–1010.
[51] E. Goncalves, E. Kitas, J. Seelig, Structural and thermodynamic aspects of the interaction between heparan sulfate and analogues of melittin, Biochemistry 45 (2006) 3086–3094.
[52] F. Duchardt, I.R. Ruttekolk, W.P. Verdurmen, H. Lortat-Jacob, J. Burck, H. Hufnagel, R. Fischer, M. van den Heuvel, D.W. Lowik, G.W. Vuister, A. Ulrich, M. de Waard, R. Brock, A cell-penetrating peptide derived from human lactoferrin with conformation-dependent uptake efficiency, J. Biol. Chem. 284 (2009) 36099–36108.
[53] A. Ziegler, J. Seelig, Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters, Biophys. J. 86 (2004) 254–263.
[54] H.L. Amand, H.A. Rydberg, L.H. Fornander, P. Lincoln, B. Norden, E.K. Esbjorner, Cell surface binding and uptake of arginine- and lysine-rich penetratin peptides in absence and presence of proteoglycans, Biochim. Biophys. Acta 1818 (2012) 2669–2678.
[55] C. Bechara, M. Pallerla, Y. Zaltsman, F. Burlina, I.D. Alves, O. Lequin, S. Sagan, Tryptophan within basic peptide sequences triggers glycosaminoglycan-dependent endocytosis, FASEB J. 27 (2012) 738–749.
[56] A.D. Cardin, H.J. Weintraub, Molecular modeling of protein–glycosaminoglycan interactions, Arteriosclerosis 9 (1989) 21–32.
[57] R. Wallbrecher, W.P. Verdurmen, S. Schmidt, P.H. Bovee-Geurts, F. Broecker, A. Reinhardt, T.H. van Kuppevelt, P.H. Seeberger, R. Brock, The stoichiometry of peptide–heparan sulfate binding as a determinant of uptake efficiency of cell-penetrating peptides, Cell. Mol. Life Sci. (2013) [Epub ahead of print].
[58] J.D. Esko, T.E. Stewart, W.H. Taylor, Animal cell mutants defective in glycosaminoglycan biosynthesis, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 3197–3201.
[59] M. Tyagi, M. Rusnati, M. Presta, M. Giacca, Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans, J. Biol. Chem. 276 (2001) 3254–3261.
[60] S. Console, C. Marty, C. Garcia-Echeverria, R. Schwendener, K. Ballmer-Hofer, Antennapedia and HIV TAT "protein transduction domains" promote endocytosis of high Mr cargo upon binding to cell surface glycosaminoglycans, J. Biol. Chem. 278 (2003) 35109–35114.
[61] W.P. Verdurmen, R. Wallbrecher, S. Schmidt, J. Eilander, P. Bovee-Geurts, S. Fanghanel, J. Burck, P. Wadhwani, A.S. Ulrich, R. Brock, Cell surface clustering of heparan sulfate proteoglycans by amphipathic cell-penetrating peptides does not contribute to uptake, J. Control. Release 170 (2013) 83–91.
[62] C.Y. Jiao, D. Delaroche, F. Burlina, I.D. Alves, G. Chassaing, S. Sagan, Translocation and endocytosis for cell-penetrating peptide internalization, J. Biol. Chem. 284 (2009) 33957–33965.
[63] A. Subrizi, E. Tuominen, A. Bunker, T. Rog, M. Antopolsky, A. Urtti, 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 (2012) 277–285.
[64] I.D. Alves, C. Bechara, A. Walrant, Y. Zaltsman, C.Y. Jiao, S. Sagan, Relationships between membrane binding, affinity and cell internalization efficacy of a cell-penetrating peptide: penetratin as a case study, PLoS One 6 (2011) e24096.
[65] M. Fotin-Mleczek, S. Welte, O. Mader, F. Duchardt, R. Fischer, H. Hufnagel, P. Scheurich, R. Brock, Cationic cell-penetrating peptides interfere with TNF signaling by induction of TNF receptor internalization, J. Cell Sci. 118 (2005) 3339–3351.
[66] I.M. Kaplan, J.S. Wadia, S.F. Dowdy, Cationic TAT peptide transduction domain enters cells by macropinocytosis, J. Control. Release 102 (2005) 247–253.
[67] A. Ziegler, J. Seelig, Contributions of glycosaminoglycan binding and clustering to the biological uptake of the nonamphipathic cell-penetrating peptide WR9, Biochemistry 50 (2011) 4650–4664.
[68] K. Padari, K. Koppel, A. Lorents, M. Hallbrink, M. Mano, M.C. Pedroso de Lima, M. Pooga, S4(13)-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cells, Bioconjug. Chem. 21 (2010) 774–783.
[69] A. Ziegler, J. Seelig, Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds, Biophys. J. 94 (2008) 2142–2149.
[70] A. Rullo, J. Qian, M. Nitz, Peptide–glycosaminoglycan cluster formation involving cell penetrating peptides, Biopolymers 95 (2011) 722–731.
[71] E. Dupont, A. Prochiantz, A. Joliot, Identification of a signal peptide for unconventional secretion, J. Biol. Chem. 282 (2007) 8994–9000.
[72] F. Burlina, S. Sagan, G. Bolbach, G. Chassaing, Quantification of the cellular uptake of cell-penetrating peptides by MALDI-TOF mass spectrometry, Angew. Chem. Int. Ed Engl. 44 (2005) 4244–4247.

[73] W.P. Verdurmen, M. Thanos, I.R. Ruttekolk, E. Gulbins, R. Brock, Cationic cell-penetrating peptides induce ceramide formation via acid sphingomyelinase: implications for uptake, J. Control. Release 147 (2010) 171–179.

[74] O. Maniti, E. Blanchard, G. Trugnan, A. Lamaziere, J. Ayala-Sanmartin, Metabolic energy-independent mechanism of internalization for the cell penetrating peptide penetratin, Int. J. Biochem. Cell Biol. 44 (2012) 869–875.

[75] A. Mishra, G.H. Lai, N.W. Schmidt, V.Z. Sun, A.R. Rodriguez, R. Tong, L. Tang, J. Cheng, T.J. Deming, D.T. Kamei, G.C. Wong, Translocation of HIV TAT peptide and analogues induced by multiplexed membrane and cytoskeletal interactions, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 16883–16888.

[76] S. Yesylevskyy, S.J. Marrink, A.E. Mark, Alternative mechanisms for the interaction of the cell-penetrating peptides penetratin and the TAT peptide with lipid bilayers, Biophys. J. 97 (2009) 40–49.

[77] J. Imamura, Y. Suzuki, K. Gonda, C.N. Roy, H. Gatanaga, N. Ohuchi, H. Higuchi, Single particle tracking confirms that multivalent Tat protein transduction domain-induced heparan sulfate proteoglycan cross-linkage activates Rac1 for internalization, J. Biol. Chem. 286 (2011) 10581–10592.

[78] T. Letoha, A. Keller-Pinter, E. Kusz, C. Kolozsi, Z. Bozso, G. Toth, C. Vizler, Z. Olah, L. Szilak, Cell-penetrating peptide exploited syndecans, Biochim. Biophys. Acta 1798 (2010) 2258–2265.

[79] J.C. Mai, H. Shen, S.C. Watkins, T. Cheng, P.D. Robbins, Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate, J. Biol. Chem. 277 (2002) 30208–30218.

[80] R.W. Mahley, Z.S. Ji, Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E, J. Lipid Res. 40 (1999) 1–16.

[81] Y. Liu, M. Jones, C.M. Hingtgen, G. Bu, N. Laribee, R.E. Tanzi, R.D. Moir, A. Nath, J.J. He, Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands, Nat. Med. 6 (2000) 1380–1387.

[82] A.H. van Asbeck, A. Beyerle, H. McNeill, P.H. Bovee-Geurts, S. Lindberg, W.P. Verdurmen, M. Hallbrink, U. Langel, O. Heidenreich, R. Brock, Molecular parameters of siRNA-cell penetrating peptide nanocomplexes for efficient cellular delivery, ACS Nano 7 (2013) 3797–3807.

[83] K. Ezzat, H. Helmfors, O. Tudoran, C. Juks, S. Lindberg, K. Padari, S. El-Andaloussi, M. Pooga, U. Langel, Scavenger receptor-mediated uptake of cell-penetrating peptide nanocomplexes with oligonucleotides, FASEB J. 26 (2012) 1172–1180.

[84] M. Kosuge, T. Takeuchi, I. Nakase, A.T. Jones, S. Futaki, Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans, Bioconjug. Chem. 19 (2008) 656–664.

[85] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12349–12354.

[86] C.K. Payne, S.A. Jones, C. Chen, X. Zhuang, Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands, Traffic 8 (2007) 389–401.

[87] B.C. Thompson, C.R. Segarra, O.L. Mozley, O. Daramola, R. Field, P.R. Levison, D.C. James, Cell line specific control of polyethylenimine-mediated transient transfection optimized with "design of experiments" methodology, Biotechnol. Prog. 28 (2012) 179–187.

[88] Z.U. Rehman, K.A. Sjollema, J. Kuipers, D. Hoekstra, I.S. Zuhorn, Nonviral gene delivery vectors use syndecan-dependent transport mechanisms in filopodia to reach the cell surface, ACS Nano 6 (2012) 7521–7532.

[89] O.N. MJ, J. Guo, C. Byrne, R. Darcy, O.D. CM, Mechanistic studies on the uptake and intracellular trafficking of novel cyclodextrin transfection complexes by intestinal epithelial cells, Int. J. Pharm. 413 (2011) 174–183.

[90] D. Vercauteren, M. Piest, L.J. van der Aa, M. Al Soraj, A.T. Jones, J.F. Engbersen, S.C. De Smedt, K. Braeckmans, Flotillin-dependent endocytosis and a phagocytosis-like mechanism for cellular internalization of disulfide-based poly(amido amine)/DNA polyplexes, Biomaterials 32 (2011) 3072–3084.

[91] P.M. McLendon, D.J. Buckwalter, E.M. Davis, T.M. Reineke, Interaction of poly(glycoamidoamine) DNA delivery vehicles with cell-surface glycosaminoglycans leads to polyplex internalization in a manner not solely dependent on charge, Mol. Pharm. 7 (2010) 1757–1768.

[92] M. Ruponen, S. Ronkko, P. Honkakoski, J. Pelkonen, M. Tammi, A. Urtti, Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes, J. Biol. Chem. 276 (2001) 33875–33880.

[93] Z. Ziraksaz, A. Nomani, M. Ruponen, M. Soleimani, M. Tabbakhian, I. Haririan, Cell-surface glycosaminoglycans inhibit intranuclear uptake but promote post-nuclear processes of polyamidoamine dendrimer-pDNA transfection, Eur. J. Pharm. Sci. 48 (2012) 55–63.

[94] C. Pramfalk, J. Lanner, M. Andersson, E. Danielsson, C. Kaiser, I.M. Renstrom, M. Warolen, S.R. James, Insulin receptor activation and down-regulation by cationic lipid transfection reagents, BMC Cell Biol. 5 (2004) 7.

[95] L.C. Mounkes, W. Zhong, G. Cipres-Palacin, T.D. Heath, R.J. Debs, Proteoglycans mediate cationic liposome–DNA complex-based gene delivery *in vitro* and *in vivo*, J. Biol. Chem. 273 (1998) 26164–26170.

[96] I. Kopatz, J.S. Remy, J.P. Behr, A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin, J. Gene Med. 6 (2004) 769–776.

[97] S. Paris, A. Burlacu, Y. Durocher, Opposing roles of syndecan-1 and syndecan-2 in polyethyleneimine-mediated gene delivery, J. Biol. Chem. 283 (2008) 7697–7704.

[98] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K.A. Dawson, S. Linse, Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 2050–2055.

[99] M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, K.A. Dawson, Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14265–14270.

[100] D. Shukla, P.G. Spear, Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry, J. Clin. Invest. 108 (2001) 503–510.

[101] M.J. Oh, J. Akhtar, P. Desai, D. Shukla, A role for heparan sulfate in viral surfing, Biochem. Biophys. Res. Commun. 391 (2010) 176–181.

[102] S. Bacsa, G. Karasneh, S. Dosa, J. Liu, T. Valyi-Nagy, D. Shukla, Syndecan-1 and syndecan-2 play key roles in herpes simplex virus type-1 infection, J. Gen. Virol. 92 (2011) 733–743.

[103] I. Mondor, S. Ugolini, Q.J. Sattentau, Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans, J. Virol. 72 (1998) 3623–3634.

[104] A.C. Saphire, M.D. Bobardt, Z. Zhang, G. David, P.A. Gallay, Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages, J. Virol. 75 (2001) 9187–9200.

[105] L. de Witte, M. Bobardt, U. Chatterji, G. Degeest, G. David, T.B. Geijtenbeek, P. Gallay, Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 19464–19469.

[106] G. Roderiquez, T. Oravecz, M. Yanagishita, D.C. Bou-Habib, H. Mostowski, M.A. Norcross, Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120–gp41, J. Virol. 69 (1995) 2233–2239.

[107] M. Sapp, M. Bienkowska-Haba, Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus, FEBS J. 276 (2009) 7206–7216.

[108] Z. Surviladze, A. Dziduszko, M.A. Ozbun, Essential roles for soluble virion-associated heparan sulfonated proteoglycans and growth factors in human papillomavirus infections, PLoS Pathog. 8 (2012) e1002519.

[109] Y. Chen, T. Maguire, R.E. Hileman, J.R. Fromm, J.D. Esko, R.J. Linhardt, R.M. Marks, Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate, Nat. Med. 3 (1997) 866–871.

[110] K. Okamoto, H. Kinoshita, C. Parquet Mdel, M. Raekiansyah, D. Kimura, K. Yui, M.A. Islam, F. Hasebe, K. Morita, Dengue virus strain DEN2 16681 utilizes a specific glycochain of syndecan-2 proteoglycan as a receptor, J. Gen. Virol. 93 (2012) 761–770.

[111] M. Kalia, V. Chandra, S.A. Rahman, D. Sehgal, S. Jameel, Heparan sulfate proteoglycans are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection, J. Virol. 83 (2009) 12714–12724.

[112] N. Kapur, D. Thakral, H. Durgapal, S.K. Panda, Hepatitis E virus enters liver cells through receptor-dependent clathrin-mediated endocytosis, J. Viral Hepat. 19 (2012) 436–448.

[113] A. Wittrup, S.H. Zhang, G.B. ten Dam, T.H. van Kuppevelt, P. Bengtson, M. Johansson, J. Welch, M. Morgelin, M. Belting, ScFv antibody-induced translocation of cell-surface heparan sulfate proteoglycan to endocytic vesicles: evidence for heparan sulfate epitope specificity and role of both syndecan and glypican, J. Biol. Chem. 284 (2009) 32959–32967.

[114] T.H. van Kuppevelt, M.A. Dennissen, W.J. van Venrooij, R.M. Hoet, J.H. Veerkamp, 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 (1998) 12960–12966.

[115] E. Saxon, C.R. Bertozzi, Cell surface engineering by a modified Staudinger reaction, Science 287 (2000) 2007–2010.

[116] J. Dommerholt, S. Schmidt, R. Temming, L.J. Hendriks, F.P. Rutjes, J.C. van Hest, D.J. Lefeber, P. Friedl, F.L. van Delft, Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells, Angew. Chem. Int. Ed. Engl. 49 (2010) 9422–9425.

[117] J. Herz, D.K. Strickland, LRP: a multifunctional scavenger and signaling receptor, J. Clin. Invest. 108 (2001) 779–784.

[118] J. Tabernero, G.I. Shapiro, P.M. LoRusso, A. Cervantes, G.K. Schwartz, G.J. Weiss, L. Paz-Ares, D.C. Cho, J.R. Infante, M. Alsina, M.M. Gounder, R. Falzone, J. Harrop, A.C. White, I. Toudjarska, D. Bumcrot, R.E. Meyers, G. Hinkle, N. Svrzikapa, R.M. Hutabarat, V.A. Clausen, J. Cehelsky, S.V. Nochur, C. Gamba-Vitalo, A.K. Vaishnaw, D.W. Sah, J.A. Gollob, H.A. Burris III, First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement, Cancer Discov. 3 (2013) 406–417.

[119] T.S. Zimmermann, A.C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornlocher, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, Nature 441 (2006) 111–114.

[120] D. Huhn, K. Kantner, C. Geidel, S. Brandholt, I. De Cock, S.J. Soenen, P. Rivera Gil, J.M. Montenegro, K. Braeckmans, K. Mullen, G.U. Nienhaus, M. Klapper, W.J. Parak, Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge, ACS Nano 7 (2013) 3253–3263.

[121] S.E. El-Andaloussi, T. Lehto, I. Mager, K. Rosenthal-Aizman, I.I. Oprea, O.E. Simonson, H. Sork, K. Ezzat, D.M. Copolovici, K. Kurrikoff, J.R. Viola, E.M. Zaghloul, R. Sillard, H.J. Johansson, F. Said Hassane, P. Guterstam, J. Suhorutsenko, P.M. Moreno, N. Oskolkov, J. Halldin, U. Tedebark, A. Metspalu, B. Lebleu, J. Lehtio, C.I. Smith, U. Langel, Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*, Nucleic Acids Res. 39 (2011) 3972–3987.

[122] F. Perche, V.P. Torchilin, Recent trends in multifunctional liposomal nanocarriers for enhanced tumor targeting, J. Drug Deliv. 2013 (2013) 705265.