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Cell-penetrating compounds preferentially bind glycosaminoglycans over plasma membrane lipids in a charge density- and stereochemistry-dependent manner

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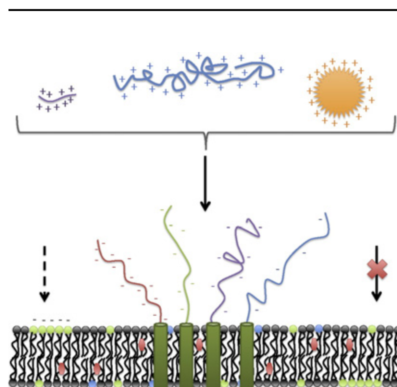
HIGHLIGHTS

- GAG charge density affects CPC binding strength.
- GAG carbohydrate composition also plays a role in CPC recognition, likely through hydrogen bonding.
- The molecular weight of CPCs affects their ability to cluster GAGs.
- CPCs bind anionic lipids but not typical plasma membrane compositions.

GRAPHICAL ABSTRACT



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ABSTRACT

Cell-penetrating compounds (CPCs) are often conjugated to drugs and genes to facilitate cellular uptake. We hypothesize that the electrostatic interaction between the positively charged amines of the cell-penetrating compounds and the negatively charged glycosaminoglycans (GAGs) extending from cell surfaces is the initiating step in the internalization process. The interactions of generation 5 PAMAM dendrimer, Tat peptide and 25 kDa linear PEI with four different GAGs have been studied using isothermal titration calorimetry to elucidate structure–function relationships that could lead to improved drug and gene delivery methods to a wide variety of cell types. Detailed thermodynamic analysis has determined that CPC-GAG binding constants range from 8.7×10^3 to 2.4×10^6 M^{-1} and that affinity is dependent upon GAG charge density and stereochemistry and CPC molecular weight. The effect of GAG composition on affinity is likely due to hydrogen bonding between CPC amines and amides and GAG hydroxyl and amine groups. These results were compared to the association of CPCs with lipid vesicles of varying composition as model plasma membranes to finally clarify the relative importance of each cell surface component in initial cell recognition. CPC-lipid affinity increases with anionic lipid content, but GAG affinity is higher for all cell-penetrating compounds, confirming the role these heterogeneous polysaccharides play in cellular association and clustering.

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1. Introduction

Cell-penetrating compounds (CPCs) are a class of molecules with the remarkable ability to cross a wide variety of cell membranes and can carry covalently or non-covalently attached cargo, such as drugs, proteins or nucleic acids with them. Some examples of CPCs include the cationic peptides Tat and penetratin, cationic polymers polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimer and cationic lipids such as 1,2-bis(oleoyloxy)-3-(trimethylammonium) propane (DOTAP) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). Although their practical usefulness in drug and gene delivery has been exploited for over 25 years [1–9], their mechanism of cellular uptake is still debated. Hypotheses generally fall into one of three classes: direct penetration of the membrane, endocytosis or entry via formation of a micelle with the plasma membrane lipids [10,11]. Direct penetration was widely considered the prominent mechanism due to experimental evidence of energy-independent uptake of CPCs; however, it was recently discovered that cell fixation artifacts had led to those conclusions [10,12–14]. Clathrin-dependent endocytosis has been suggested as a possible mode of CPC cellular entry due to microscopy evidence of their vesicle containment once internalized and their decreased uptake upon cell treatment with specific endocytosis inhibitors [15,16]. The important conclusion from these studies is that these mechanisms may not be mutually exclusive and may depend on whether or not cargo is attached to the CPC [17–19].

The structures of CPCs vary, but they all have one characteristic in common, high positive charge density, which leads to the hypothesis that their cell penetrating properties are related to electrostatic interactions at the cell surface. Cell plasma membranes are composed of anionic and zwitterionic lipids and proteoglycans presenting highly sulfated linear polysaccharides called glycosaminoglycans into the extracellular milieux. Since both of these classes of molecules are universal to all cell types, they are both possible cell surface recognition elements for CPCs. Electrostatic interactions could serve to temporarily disrupt the plasma membrane allowing for direct penetration [20–22] or concentrate the CPCs at receptors to trigger endocytotic mechanisms.

Interactions between CPCs and plasma membrane lipids, usually in the form of small or large unilamellar vesicles (SUVs or LUVs, respectively), have been documented [23]. Amine-terminated PAMAM dendrimers were simulated interacting with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) lipid bilayers with free energy values of -50 and -110 kcal/mol, respectively [24]. Tiriveedhi et al. made a similar conclusion, that membrane interactions of PAMAM dendrimers increase as a function of anionic lipid content [$K_D =$ 30 μM (100% PC) and 11 μM (3:1 PC:PG lipid molar ratio)] [25], based on fluorescence anisotropy and surface tensiometry experimental data and also showed that higher dendrimer generations had greater membrane association [26].

Cell-penetrating peptides also show lipid membrane affinities that are dependent upon anionic lipid content. The dissociation constant, K_D, for penetratin's interaction with lipid vesicles is 63 μM for 9:1 PC:PG lipid molar ratio [28] and 0.22 μM for 7:3 ratio [29]. Tat peptide shows a similar trend with $K_D = 83$ μM (3:1 ratio) and 9.1 μM (1:1 ratio) [30]. Comparing Tat and penetratin interactions with similar lipid content vesicles shows that unstructured Tat has weaker affinity than penetratin, which rearranges from disordered to a β-sheet upon phospholipid binding. Eiriksdottir et al. compiled data on many different classes of peptides and found that the ability of peptides to adopt specific conformations is an important factor governing lipid membrane binding [27].

To date, there are no reported dissociation constants for PEI's interaction with model cell membranes. Using sum frequency generation (SFG) and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy, Zhang et al. recently showed that PEI induced lipid translocation in both 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) supported lipid bilayers in a concentration-dependent manner [31]. They concluded that interactions were weaker for zwitterionic lipid head groups based on the lipid translocation rate. Classical molecular dynamics simulations of linear PEI and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayers showed pore formation due to the polycation settling at the bilayer–water interface, but they did not measure a dissociation constant or compare to an anionic lipid model [32].

In addition to this documented plasma membrane lipid binding, it has been shown that both down-regulating expression and enzymatic removal of glycosaminoglycans from cell surfaces significantly reduce internalization of CPCs [12,33,34]. This suggests that GAGs play a role in cellular uptake of these materials; however, the nature of the role and the structure–function relationships involved still need elucidating. Such studies are especially important but challenging given that GAG fine structure is highly regulated by cells. Glycosaminoglycans are generally composed of a glycosamine (N-acetylglucosamine or N-acetylgalactosamine) O-linked to an uronic acid (iduronic acid or glucuronic acid). Epimerization of glucuronic acid to iduronic acid is regulated by the enzyme glucuronyl C5-epimerase. Sulfotransferases and deacetylases control the charge density of the GAGs through the 6-O and N of the glycosamine and the 2-O of the uronic acid. Such dynamic modifications result in the structures most commonly found on cell surfaces: heparan sulfate (HS), chondroitin sulfate A (CSA), and dermatan sulfate (DS) all shown in Fig. 2. The most prevalent repeat units of CSA and DS have the same charge density (only N-sulfated) but differ in the type of uronic acid (glucuronic acid for CSA and iduronic acid for DS). Heparan sulfate consists of repeats of more charge-dense N-sulfated domains with iduronic acid separated by long segments of low charge-dense N-acetylated domains with a higher composition of glucuronic acid. While heparan sulfate is on the surface of most animal cells, chondroitin sulfate A is usually found in cartilage tissue and bone, and dermatan sulfate is associated with heart valves, skin, tendons and lungs [35,36]. Heparin is the most sulfated of the GAGs (N-, 6-O- and 2-O-sulfated) and is the most charge dense molecule in nature. It is often prescribed as an anticoagulant and is thought to play a role in cell defense. Although not found on cell surfaces [37], it is the most common GAG for binding studies, used as a model for the highly sulfated domains of heparan sulfate.

Binding between cell-penetrating peptides and heparin has been investigated experimentally, and dissociation constants ranging from 0.459 μM for R_9 to 0.338 μM for penetratin and 0.443 μM for Tat were found [38]. The spatial arrangement of positive charge density on peptides plays an important role, with α-helices with all charges displayed on one side of the helix demonstrating significantly higher heparin affinity than those with charges randomly positioned [39]. Clustering of peptide–heparin complexes, thought to be a prerequisite for cellular uptake, was also studied using dynamic light scattering and fluorescence quenching assays by Mark Nitz's group [40]. They found that clustering was a reversible process driven by nonpolar contacts between the peptides and that peptide structure determined stability of these aggregates. The interaction of Tat peptide with other GAGs was studied using isothermal titration calorimetry by Ziegler and Seelig who found very similar affinities for heparin, DS and HS ($K_D = 1.7$, 4.0 and 1.7 μM, respectively) on a per sulfate basis [41]. The DS and HS used here were of similar sulfur content (~6% by mass), while the heparin was more charge-dense (~10% sulfur) as expected.

Much less work has been done to determine the affinity of cationic polymers for glycosaminoglycans. Linear 2.5 kDa PEI was shown to bind heparin with a K_D of 0.741 μM at pH 7.4, which decreased to 0.531 μM at pH 5.0 [38]. Unfortunately, this PEI is much shorter than that used to deliver genes and drugs, and molecular weight may play a role in binding. The only other relevant work involves the competitive displacement of DNA from branched 25 and 800 kDa PEI and fractured G6 PAMAM dendrimer polyplexes by GAGs using relative fluorescence of DNA-intercalated ethidium bromide and gel electrophoresis [42].

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