Affinity Capillary Electrophoresis in Pharmaceutics and Biopharmaceutics

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Characterization of Polysaccharide Interactions

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I. INTRODUCTION

In recent years, much attention has been paid to the chemistry and biochemistry of carbohydrates due to their many functions both inside and outside living cells. The glycosaminoglycans (GAGs) are related linear, polydisperse, microheterogeneous polyanionic polysaccharides. The most common GAGs are heparin, heparan sulfate, hyaluronic acid, chondroitin sulfate, dermatan sulfate, and keratan sulfate. The GAGs are generally believed to exert their biological activities through the localization, stabilization, activation, or inactivation of interacting proteins (1). These interactions play important roles in the normal physiology of animals (2) and are also involved in certain pathological processes (3). Heparin, the most studied GAG, is unique in its intracellular location in mast cell and basophil granules. Exogenous heparin is regularly used as an anticoagulant/antithrombotic agent to maintain blood flow in the vasculature through the binding and activation of antithrombin III (ATIII, a coagulation serine protease inhibitor, SERPIN) (1). Heparin has been found to bind a wide range of proteins (1,4), and it regulates a number of important biological activities. Heparan sulfate, structurally similar to heparin, is localized on the external surface of cell membranes and in the extracellular matrix and plays a major role in cell–cell and cell–protein interaction (1). Heparan sulfate (not heparin) is also believed to be an endogenous receptor for circulating growth factors and chemokines that reg-
ulate cell growth and migration (1). Other GAGs also bind proteins, but the interactions are generally less well studied than those of heparin and heparan sulfate. In addition to GAGs there are a number of synthetic, semisynthetic, and natural GAG analogues that are of medicinal interest (1). An understanding of how these GAGs and GAG analogues interact with proteins is needed to develop either carbohydrate-based, peptide-based, or synthetic therapeutics for the prevention and treatment of disease processes, including control of coagulation (1) and the control of tumor cell replication, migration, invasion, and vascularization (2,3). In this chapter, the structures and therapeutic potential of these important GAGs, GAG-binding proteins, and the quantitative methods for characterization of polysaccharide–protein binding interactions will be provided as a background. Recent advances in the application of affinity capillary electrophoresis (ACE) to study these specific interactions will be discussed.

II. DEFINITION OF OLIGOSACCHARIDES, POLYSACCHARIDES, GLYCOSAMINOGLYCANS, AND PROTEOGLYCANS

Carbohydrates are classified as monosaccharides, oligosaccharides, or polysaccharides based on their number of monomer units.

_Glycosaminoglycans (GAGs)_ are a family of complex linear anionic polysaccharides found in most animal tissues. The polysaccharides are characterized by a repeating core disaccharide structure typically composed of uronic acid and hexosamine residues. In GAGs, the amino group of the hexosamine residue is either N-acetylated or N-sulfonated; the uronic acid may be either D-glucuronic acid or L-iduronic acid. Moreover, the repeating disaccharide units (i.e., uronic acid–hexosamine) are O-sulfonated to varying degrees at the 6- and/or 4-position of the various hexosamine residues and at the 2-position of the uronic acid residues. Heparin, heparan sulfate, hyaluronic acid, chondroitin sulfate, dermatan sulfate, and keratan sulfate are the most common GAGs.

_Proteoglycans (PGs)_ are a diverse group of proteins unified by their possession of one or more covalently attached GAG chains. With the exception of hyaluronic acid, GAGs are biosynthesized as proteoglycans (1). Some PGs, such as heparin, are biochemically processed into GAGs through the action of proteinases and β-endoglucuronidases (1) (Fig. 1). Thus, both PGs and GAGs are natural components of most animal tissues.
Fig. 1  Structure of heparin proteoglycan. The heparin proteoglycan is released from mast cells on degranulation as raw heparin formed through the action of β-endoglucuronidase and proteinase. (a) Raw heparin can be separated (b) into peptidoglycan heparin and glycosaminoglycan (GAG) heparin. Pharmaceutical heparin is prepared by bleaching raw heparin to remove peptide. (Adapted from Ref. 1.)
III. IMPORTANT POLYSACCHARIDES

A. Heparin

Heparin is biosynthesized in mast cells as a PG with approximately 10 GAG side chains (5). When mast cells degranulate, PG heparin is enzymatically degraded to GAG heparin (Fig. 1). Exogenous heparin is used primarily as an anticoagulant/antithrombotic agent. Heparin is produced from mast-cell-rich tissues, such as lung and intestinal mucosa, in metric ton quantities yearly for use as a pharmaceutical agent.

Heparin is a repeating linear copolymer of 1 → 4 linked uronic acid and glucosamine residues (6). The uronic acid residue typically consists of 90% iduronic acid and 10% glucuronic acid. Iduronate is a more flexible residue than glucuronate, promoting polysaccharide interaction with proteins. Heparin has a high negative charge density, the result of sulfo and carboxyl groups that are present in its structure. Indeed, the average disaccharide in heparin contains 2.7 sulfo groups. Whereas the most common structure in heparin is the disaccharide containing 3 sulfo groups (7) (Fig. 2), a number of structural variants exist, making it microheterogenous (8). The 2-position of uronic acid residue frequently contains an O-sulfo group. The 6-position of the glucosamine residue often contains an O-sulfo group while the 3-position infrequently contains an O-sulfo group, and the amino functionality at the 2-position of the glucosamine residue may contain an N-sulfo group or N-acetyl group or be unsubstituted. The presence or absence of these functional groups, as well as the presence of two different uronic acid residues (iduronate and glucuronate), define the sequence of the GAG chain. The length of heparin GAG chains can also vary. Heparin is polydisperse; the average chain in a commercial heparin has a molecular weight of 13,000, with chains ranging in size from 5,000 to 40,000.

Because of its high content of sulfo groups and its sequence microheterogeneity, heparin is able to bind a wide range of proteins and regulate a number of important biological activities (1). These interactions are currently under intensive investigation, and they regulate heparin’s effect on lipoprotein lipase, on smooth muscle proliferation, its inhibition of complement activation, anti-inflammatory activity, angiogenic and antiangiogenic activities, anticancer activity, antiviral activity, and its potential use in treating Alzheimer’s disease (1).

As a result of heparin’s multiplicity of biological activities and its importance as a major pharmaceutical, other polysaccharides and modified polysaccharides have been examined as potential heparin analogues in drug development (1). These heparin analogues include other GAGs, other non-GAGs, sulfated polysaccharides from plant and animal origins such as lam-
inarin and acharan sulfate, and synthetic analogues of heparin such as subaramin (Fig. 3).

B. Low-Molecular-Weight Heparins

Low-molecular-weight (LMW) heparins are generally prepared through the controlled, partial, chemical or enzymatic depolymerization of commercial GAG heparin (1). Therefore, they are primarily composed of the same small number of major oligosaccharide sequences that make up standard commercial heparins. The LMW heparins are polydisperse mixtures (average molecular weight 4,000–8,000) that have proven to be effective antithrombotic agents (9) for use in a wide variety of disease states, ranging from deep vein thrombosis (10) to nonhemorrhagic stroke (11). The LMW heparins offer several advantages over the anticoagulant heparin (1). They are as safe as heparin, resulting in somewhat less hemorrhagic complications, and they have greater bioavailability and longer half-lives, resulting in better dose control (11). These potential advantages have led to the development of several commercial LMW heparin preparations.

C. Heparin Analogues

There are a number of structural analogues of heparin that have been and are being evaluated as therapeutic agents (Table 1). These analogues include polysaccharide natural products, chemically modified polysaccharides, and synthetic molecules containing anionic groups, including N-sulfo groups, O-sulfo groups, sulfonyl groups, carboxyl groups, and phosphoryl groups. As with heparin, these analogues exert their activities by interacting with various heparin-binding proteins.

IV. THERAPEUTIC POTENTIAL OF POLYSACCHARIDES

Heparin and heparin analogues are used primarily as anticoagulant and antithrombotic agents to augment the activity of ATIII, resulting in the inactivation of coagulation proteases and blood anticoagulation. Heparin and LMW heparins are the most commonly used clinical anticoagulants. However, there are additional therapeutic areas that are under extensive investigation (Table 2). Some of these are described next in detail.
Heparin

X = H or SO_3, Y = H, CH_3CO or SO_3

Heparan sulfate

X = H or SO_3, Y = H, CH_3CO or SO_3

major disaccharide sequence

minor disaccharide sequence
Fig. 2 Structures of the major and minor disaccharide sequences of heparin and heparan sulfate as well as the major disaccharide units of chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid, where Ac is acetyl.
A. Regulation of Enzymatic Processes

Since this is the major current application for heparin as an anticoagulant/antithrombotic, it is not surprising that much of the research has targeted this type of activity. The extensive evaluation of heparin analogues as anticoagulant/antithrombotic agents has met with some success, including the clinical introduction of LMW heparins and pentosan polysulfate (1,32,33). Recently, heparin analogues have been evaluated as regulators of the complement cascade and proteases, such as elastase, for the treatment of lung disorders, such as hereditary angioedema (48) and asthma (49).

B. Anti-Infection

It is clear that pathogens use endogenous GAGs to localize on the surface of cells and even to gain entrance into cells, resulting in infection (50). The increasing number of reports of antibiotic-resistant microorganisms suggest that new anti-infective mechanisms, such as ones disrupting cellular localization of pathogens, might be valuable. The dengue virus causing hemorrhagic fever in many tropical countries is an example of an infectious disease for which there is no vaccine available and no known specific treatment. An oversulfonated heparan sulfate was shown to be a receptor required for infectivity by this virus (47). In addition, heparin and a heparin decasaccharide (47) were demonstrated to inhibit virus infectivity through their blocking effects at both virus attachment and penetration (51). Interestingly, a heparin analogue, suramin, can also block such infection in vitro (47). Recently, GAGs and their derivatives have been examined as potential inhibitors of dengue virus envelope protein binding to its receptors (52). The molecular size and level of sulfation of GAGs were reported to affect its inhibitory activity.

C. Anticancer

Cancer is a complicated process consisting of well-coordinated multiple steps. Randomized trials to study the effectiveness of LMW heparins as compared with unfractionated heparin in treating venous thromboembolism in cancer patients led to a surprising observation that treatment with heparin

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Fig. 3 Structures of acharan sulfate, dextran sulfate, suramin, pentosan polysulfate, and phosphomannopentaose sulfate (PI-88). Representative saccharide units comprising each of these polymers are shown, where $n$ is the degree of polymerization.
<table>
<thead>
<tr>
<th>Analogue</th>
<th>Structure</th>
<th>Current applications</th>
<th>Remarks</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparan sulfate</td>
<td>→4) glucuronic acid (1 → 4) (N)-acetyl glucosamine (1 → &lt;1) sulfo group/disaccharide</td>
<td>Component of antithrombotic agents</td>
<td>High price and limited availability</td>
<td>6, 8</td>
</tr>
<tr>
<td>Chondroitin sulfate and dermatan sulfates</td>
<td>→3) (N)-acetyl galactosamine (1 → 4) glucuronic acid/(1 \rightarrow, with \sim 1) sulfo group</td>
<td>Antithrombotic primarily through interaction with heparin cofactor II (HCII)</td>
<td>Binds annexin and other proteins on persulfatation</td>
<td>1, 12–21</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>→3) (N)-acetyl glucose amine (1 → 4) glucuronic acid (1 \rightarrow) unsulfated; high MW galactose and (N)-acetyl glucose amine 6-O-sulfo groups</td>
<td>Vitreous humor and synovial fluid replacement</td>
<td>Binds link protein and CD44; on persulfation, affords hyaluronidase inhibitors</td>
<td>18–23</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>None known</td>
<td>None known</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Acharan sulfate</td>
<td>→4) 2-sulfo iduronic acid (1 → 4) (N)-acetyl glucosamine (1 \rightarrow)</td>
<td>May serve as an antidesiccant, metal chelator, anti-infective, or locomotive (slime) agent in snail</td>
<td>Inhibits heparin's FGF-2 mitogenic activity in vitro; N-sulfation affords unique structural analogs of heparin</td>
<td>19, 21, 25, 26</td>
</tr>
<tr>
<td>Carrageenans (\kappa), (\lambda), and (\lambda)</td>
<td>sulfo ester of (d)-galactose and 3,6-anhydro-(d)-galactose units, linked (\alpha \rightarrow 3) and (\beta \rightarrow 4) in the polymer</td>
<td>Gelling and thickening agents, pharmaceutical excipients for controlled release</td>
<td>Under in vivo evaluation as inhibitors of tumor growth</td>
<td>27–29</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>A chemically sulfated (1 → 4)-β-D-, (1 → 3)-α-D-branched glucan polymer</td>
<td>Treatment of acute respiratory distress syndrome (ARDS), lipoprotein-releasing activity, in biomaterials as nonthrombogenic surfaces</td>
<td>Inhibitor of human immunodeficiency virus (HIV) binding to T-lymphocytes</td>
<td>1, 30–31</td>
</tr>
<tr>
<td>Pentosan polysulfate</td>
<td>(1 → 4)-β-linked xylan oligosaccharide that is branched in the center with a single β-(1 → 2)-4-O-methyl-α-D-glucuronate residue</td>
<td>Antithrombotic prophylactic in Europe, for interstitial cystitis in the United States</td>
<td>Animal models to treat osteoarthritis, preclinical studies as anticancer agent, inhibits metastasis and angiogenesis</td>
<td>1, 32–37</td>
</tr>
<tr>
<td>Phosphomannopentaose sulfate (PI-88)</td>
<td>persulfated α-6-phosphomannose (1 → 3) [α-mannose (1 → 3)]β (1 → 6)-α-mannose</td>
<td>Antiangiogenic activity as a heparanase inhibitor, HCH-mediated anticoagulant</td>
<td>Preclinical studies inhibitor of tumor metastasis, tumor growth, and angiogenesis</td>
<td>38, 39</td>
</tr>
<tr>
<td>Suramin</td>
<td>polysulfonated naphthylurea</td>
<td>Anthelmintic, antiprotozoal, antineoplastic, and antiviral agent</td>
<td>Binds many proteins, i.e., cytokines, epidermal growth factor, and members of the FGF family; inhibits dengue virus infectivity of host cells; very long in vivo half-life, exhibits a wide range of toxic side effects</td>
<td>40–47</td>
</tr>
</tbody>
</table>
### Table 2  Potential Therapeutic Applications for Heparin and Heparin Analogues

<table>
<thead>
<tr>
<th>Application</th>
<th>Status</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticoagulant/antithrombotic</td>
<td>Currently used</td>
<td>1</td>
</tr>
<tr>
<td>Antiatherosclerotics</td>
<td>Clinical trials</td>
<td>58</td>
</tr>
<tr>
<td>Complement inhibitors</td>
<td>Clinical trials</td>
<td>48</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>Animal studies</td>
<td>49</td>
</tr>
<tr>
<td>Anticancer agents</td>
<td>Animal studies</td>
<td>53</td>
</tr>
<tr>
<td>Antiangiogenic agents</td>
<td>Animal studies</td>
<td>54</td>
</tr>
<tr>
<td>Antiviral agents</td>
<td>Animal studies</td>
<td>47</td>
</tr>
<tr>
<td>Anti-Alzheimers agents</td>
<td>Animal studies</td>
<td>59</td>
</tr>
<tr>
<td>Antiprion agents</td>
<td>In vitro studies</td>
<td>60</td>
</tr>
<tr>
<td>Antiparasitic agents</td>
<td>Animal studies</td>
<td>50</td>
</tr>
<tr>
<td>New biomaterials</td>
<td>Currently used</td>
<td>61</td>
</tr>
</tbody>
</table>

may affect survival of patients with malignancy (53). The hypothesis that heparins affect cancer progression is reported by many recent experimental studies (54,55). Cancer patients who had been treated with LMW heparin for their thrombosis had a slightly improved 3-month survival as compared to cancer patients receiving unfractionated heparin. Heparin can potentially exert its activity at various stages in cancer progression and malignancy-related processes. It can affect cell proliferation, interfere with the adherence of cancer cells to vascular endothelium, regulate the immune system, and have both inhibitory and stimulatory effects on angiogenesis (54). There is recent evidence showing that heparin treatment reduces tumor metastasis in mice by inhibiting P-selectin-mediated interactions of platelets with carcinoma cell-surface mucin ligands (56). Moreover, the extensive studies aimed at understanding the role of endogeneous heparan sulfate in the regulation of cellular growth has suggested the value of heparin analogues as a potential new class of therapeutic agents. Such agents could be used in wound healing and in promoting angiogenesis in the regeneration of vessels following stroke (57).

The GAGs or their analogues may also be administered to: (1) activate protein-based receptors (agonists); (2) inactivate protein-based receptors (antagonists); (3) compete with endogenous GAGs; and (4) inhibit GAG-synthesizing or -metabolizing enzymes. Thus, studies are ongoing to examine the potential for GAG-binding proteins, peptides, peptidomimetics, and analogues to prevent and treat a wide variety of disease processes.
V. INTERACTION OF POLYSACCHARIDES WITH PROTEINS

Heparin has been found to bind a large number of proteins (Table 3). The biological activity of heparin and related polysaccharides is usually ascribed to their interaction with heparin-binding proteins. These proteins can be classified into classes including: (1) enzymes, (2) protease inhibitors, (3) lipoproteins, (4) growth factors, (5) chemokines, (6) selectins, (7) extracellular matrix proteins, (8) receptor proteins, (9) viral coat proteins, (10) nuclear proteins, and (11) other proteins (1). Many heparin-binding proteins are enzymes and enzyme inhibitors. For example, proteases in the coagulation cascade, such as factors IIa, IXa, Xa, XIIa, and VIIIa, are heparin-
binding proteins. Inhibitors of the coagulation cascade, such as ATIII and HCII, as well as the enzymes and the inhibitors of other biochemical cascades, such as the complement pathway, are also heparin-binding proteins (1,13,14,48,49). Other enzymes, such as lipolytic enzymes, contain allosteric sites that are important for both the localization and the control of their activities. Important research is aimed at understanding GAG interaction with growth factors and their cellular receptors, the tyrosine kinases (62). The fibroblast growth factors (FGFs) are among the most extensively studied of the heparin-binding growth factors. Cell surface heparan sulfate PG serves as a reservoir for these growth factors. The currently held mechanism of action for FGF (Fig. 4) involves binding to a GAG side chain of heparan sulfate PG, followed by FGF dimerization. Presentation of dimerized FGF to its tyrosine kinase receptor (also a heparin-binding protein) results in receptor dimerization and autophosphorylation. This reaction begins an intracellular biochemical cascade resulting in cell replication (or growth). A single heparin chain, an oligosaccharide of sufficient length [i.e., dodeca- or tetradeca (62)], or a heparin analogue can often substitute for the GAG and stimulate growth. A number of nuclear proteins, i.e., histones, transcription factors, topoisomerase, also bind heparin (1).

The interaction of heparin-binding proteins with heparin usually involves both ionic and hydrogen-bonding interactions (4,63). Heparin is the strongest acid present in the body and, thus, is present under physiologic conditions as a highly charged polyanion (1,63). Arginine and lysine are positively charged under physiologic pH and capable of ion pairing with the O- and N-sulfo groups and carboxyl groups of heparin. Hydrogen-bonding interactions can involve basic and other polar (i.e., Asn, Gln, Ser, etc.) amino acids. Typically, ionic and hydrogen-bonding residues lie in a spatially close array on the surface or in a shallow binding pocket on the surface of the heparin-binding protein (1). While hydrophobic interactions have limited importance in heparin binding, substantial hydrophobic contributions to binding may result in the interaction of GAGs having hydrophobic character (i.e., suramin), making these exquisitely potent agents.

The saccharide backbone present in heparin and many heparin analogues present their anionic substituents (sulfo and carboxyl groups) in a complex spatial array, owing to the high level of chirality, different regioisomers, multiple conformers, and secondary structural features of the carbohydrate backbone. Moreover, the flexibility of the saccharide backbone permits reorientation of these charged groups during binding, facilitating these interactions. Heparin (and to lesser extent heparán and dermatan sulfates) contains a flexible L-iduronic acid that in many cases is essential for binding (4). Occasionally, heparin–protein interactions (i.e., selectin, annexins) even require a divalent metal, such as Ca²⁺, for binding.
Fig. 4 Interaction of basic FGF on heparan sulfate PG (1) and free GAG (2) induces FGFR dimerization, leading to tyrosine kinase (TK) activation and signal transduction.
VI. APPROACHES FOR MEASURING POLYSACCHARIDE–PROTEIN INTERACTIONS

Numerous methods are available for analyzing polysaccharide–protein interactions (63). Many are also applicable to the study of other macromolecular interactions, such as protein–protein (see Chapter 9) and protein–DNA (see Chapter 10) binding. However, because the monovalent binding of polysaccharides to proteins is often relatively low affinity, special considerations apply. For example, many interactions are most easily observed using systems that allow multivalent binding. In addition, because chemical modification of small carbohydrate ligands may significantly alter their binding properties, methods that do not involve traditional labeling techniques are favored. Methods for quantitative analysis of binding interactions can be divided into two groups: those in which one binding partner is immobilized (mixed-phase methods) and those in which both binding partners are in solution (solution-phase methods). Individual methods within these two groups differ in the type of information they afford, as well as in their experimental complexity and the amounts and types of samples needed. No single method is ideal, and a combination of complementary methods often represents the best approach for characterizing polysaccharide–protein interactions. Table 4 presents salient features of the most common methods in use today for the quantitative analysis of polysaccharide–protein binding interactions.

VII. APPLICATIONS OF AFFINITY CAPILLARY ELECTROPHORESIS IN STUDYING POLYSACCHARIDE–PROTEIN INTERACTIONS

Many papers have described methodologies useful for characterizing ligand–protein interactions (63–67). However, among these methods, ACE has been the subject of much attention recently for the evaluation of affinity interactions because of both its high resolution and the extremely small amounts of sample required.

The ACE technique characterizes the interaction between protein and ligand, since under the conditions of electrophoresis the migration of complexed species differs from the migration of free species. The differences in the migration patterns between both species in an electrical field are used to quantify and identify specific binding and to estimate the parameters of the interaction. Since the analyte mobilities are highly dependent on charge, it is evident that interactions with negatively charged ligands, such as sul-
### Table 4  Quantitative Methods for Characterization of Carbohydrate–Protein Binding Interactions

<table>
<thead>
<tr>
<th>Method</th>
<th>Type</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface plasmon resonance (SPR)</td>
<td>M</td>
<td>Mass-induced refractive index change in real time for direct measurement of association and dissociation rate constants</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>M</td>
<td>Immobilized ligand on column matrix</td>
</tr>
<tr>
<td>Competition ELISA</td>
<td>M</td>
<td>Solution-phase and solid-phase ligands compete for binding to lectin</td>
</tr>
<tr>
<td>Scintillation proximity</td>
<td>M</td>
<td>Proximity of radiolabeled ligand to immobilized lectin results in emitted photon from scintillant</td>
</tr>
<tr>
<td>Affinity coelectrophoresis (AE)</td>
<td>M/S</td>
<td>Gel retardation: one-dimensional electrophoresis of carbohydrate through protein-impregnated gel</td>
</tr>
<tr>
<td>Two-dimensional affinity resolution electrophoresis (2DARE)</td>
<td>M/S</td>
<td>A two-dimensional AE separation</td>
</tr>
<tr>
<td>Nuclear magnetic resonance (NMR)</td>
<td>S</td>
<td>Chemical shift, coupling constants, and spectroscopy nuclear Overhauser effect allows calculation of contact points, distances, and conformation</td>
</tr>
<tr>
<td>Fluorescence spectroscopy</td>
<td>S</td>
<td>Conformational change with ligand binding induces change in fluorescence properties of intrinsic or extrinsic fluorophore</td>
</tr>
<tr>
<td>Circular dichroism (CD)</td>
<td>S</td>
<td>Change in rotation of plane-polarized light upon binding to measure conformational change</td>
</tr>
<tr>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
<td>S</td>
<td>Measures protein and carbohydrate vibrational, stretching, and bending energies</td>
</tr>
<tr>
<td>Isothermal microtitration calorimetry (ITC)</td>
<td>S</td>
<td>Measures enthalpy of binding directly</td>
</tr>
<tr>
<td>Equilibrium dialysis</td>
<td>S</td>
<td>Semipermeable membrane partitions protein but not carbohydrate ligand</td>
</tr>
<tr>
<td>Analytical ultracentrifugation</td>
<td>S</td>
<td>Equilibrium sedimentation at different carbohydrate:protein ratios yields stoichiometry of complex</td>
</tr>
<tr>
<td>Laser light scattering (LLS)</td>
<td>S</td>
<td>Intrinsic scattering intensities of carbohydrate–protein complex used to calculate stoichiometry</td>
</tr>
</tbody>
</table>

M, mixed phase; S, solution phase (see text).
fated oligo- and polysaccharides, are highly suitable for characterization by this method.

The principle and theory of the ACE method is discussed in detail in Part I of this book and were recently reviewed elsewhere (68–70). The remainder of this section is focused on the recent applications of ACE for studying GAG–protein and polysaccharide–protein interactions.

A. Experimental Settings

Based on recent reports on the study of polysaccharide–protein interaction using ACE, two different interaction measurements can be distinguished: the solution-phase method and the mixed-phase method.

The interactions can be studied when both the analyte and the affinity molecule are in free solution. In most polysaccharide–protein interaction studies, the protein is injected as a substrate to the capillary and the polysaccharide is in the running buffer. The change in migration of the protein due to the binding to the polysaccharide is observed, and this allows the affinity of interaction to be determined. However, a mobility change of the polysaccharide might also be observed in cases where little or no change in protein mobility is observed.

The ACE technique has been used to screen heparin-binding sites in serum amyloid P component by observing the migration-shift patterns of this protein in the presence of varying amounts of heparin in buffer (Fig. 5) (71). Following a similar strategy, basic human lactoferrin from two different sources, neutrophil granulocytes and milk, were compared on the basis of their affinity for heparin (72). Changes in peak shape and large shifts in migration time were observed, suggesting that the two forms of lactoferrin were identical. While several interaction studies have been successfully performed based on this approach, it still has potential drawbacks for the analysis of polysaccharide–protein/peptide interactions: (1) the possibility of varying electroosmotic flow (EOF) at different concentrations of highly charged ligand (i.e., heparin) and (2) the need for a large excess of one of the binding partners.

Interactions can also be studied at the surface of a coated capillary wall. One binding partner is first immobilized on the capillary wall. As a result of the affinity of the second binding partner, the analyte will be delayed, compared with migration times observed in an untreated capillary. Based on this approach, modified capillaries have been prepared and used successfully to study polysaccharide–protein interactions as well as affinity separations. Coating of the capillary wall with heparin and heparan sulfate has been used to determine the affinity of these polysaccharides for synthetic heparin-binding peptides different only in the stereochemistry of a single
Polysaccharide Interactions

Fig. 5 Screening by affinity CE for interaction of SAP peptides with heparin in solution. An endoproteinase Asp-N-treated Glu-C digest of SAP solubilized in water was injected for 12 s and subjected to CE at 15 kV (detection at 200 nm) in the presence of heparin (Hep) (B) added to the electrophoresis buffer (0.1 M phosphate, pH 7.5) at the concentration indicated. The peptide marked with asterisks was identified by spiking with HPLC-purified fragments and corresponds to the fragment in Figure 6. (From Ref. 71.)

amino acid (upcoming Fig. 8) (73). This approach leads to a fixed concentration of heparin or heparan sulfate ligand and a constant EOF. Moreover, the use of an immobilized ligand offers the advantage of requiring smaller amounts of both ligand and ligate. However, there is still a number of drawbacks to the method. The important potential problems are that it requires additional effort to accurately determine the concentration of the immobi-
lized heparin ligand, the immobilized ligand might not be uniformly accessible for all interactions, and there is a risk of altering the ligand characteristics through the derivatization chemistry.

An ultraviolet (UV) monitor is most commonly used in CE experiment. Such interaction studies using the ACE method can also be hampered by the inadequate sensitivity of UV detection. Fluorescence labeling and laser-induced fluorescence (LIF) detection have been employed to enhance the sensitivity of this method, as shown by the mobility-shift assay of fluorescence-labeled sugar caused by the interaction with the lectin, concanavalin A (74). When fluorescent dyes are employed for labeling, LIF detection provides several hundred times more sensitivity than UV detection.

The ACE technique has also been used in pharmaceutical and medical research. Affinity interactions of drugs with interfering compounds present in the body are important parameters of the interest for drug discovery, action, and metabolism. And ACE has been applied to the analysis of polysaccharide–drug interaction. Amylopectin oligomers were used as model solutes to investigate certain carbohydrate–drug interactions (75). Fluorescently labeled oligosaccharides, used to observe the interaction of an oligosaccharide with the drug, provided a mobility shift in this ACE experiment. In an analogous procedure, interaction between different starch degradation products and the β-blocker propanolol were studied using gel permeation experiments and ACE (76). The mobility of the drug under electrophoresis is known to be altered by its aggregation with starch. A similar interaction might be responsible for the retarded transport of propanolol over the membranes in the presence of malto-oligosaccharides.

Another interesting and recent application of ACE is the fluorescence-enhanced competition assay for the detection of sugar–lectin interactions (77). Because glucose competes with the fluorescently labeled dextran for the lectin-binding sites, the relative fluorescent intensity due to the displaced dextran is proportional to the concentration of glucose.

The affinity of polysaccharides for drug has also been exploited to assist the separation of enantiomeric drugs (78,79). About 40% of drugs in the marketplace are known to be chiral (79). The pharmacological activity of these drugs is almost entirely restricted to one of the enantiomers. In several cases, unwanted side effects or even toxic effects have been caused by the second, inactive enantiomer. Therefore, the development of methods for enantiomeric separation is of increasing interest. Ionic and neutral polysaccharides have been shown to be useful in such separation of basic drugs. The fundamental aspects and applications of these separations are discussed in detail elsewhere (80,81). Cyclodextrins are the most frequently used chiral selectors (79) (see also Chapter 8). Negatively charged polysaccharides such as heparin, chondroitin sulfate A and C, dextran sulfate, and λ-carrageenan
have also been used as chiral selectors for the separation of basic drugs (80,81). Recently, several new GAGs have been investigated as chiral selectors: Dermatan sulfate has been successfully demonstrated to act as an enantiomeric selector for a variety of basic drugs, such as β-sympathomimetics, β-blockers, and antihypertensives (82). More recently, pentosan polysulfate was also investigated as a chiral selector for tryptophan derivatives and several drugs (83).

B. Recent Examples

1. Example 1: Using ACE to Determine the Heparin-Binding Site in Proteins

Based on the solution-phase approach, ACE was used to identify and characterize heparin-binding peptides derived from serum amyloid P (SAP) (71). The protein was digested by proteolytic enzyme, Glu-C endoproteinase. The peptides formed on digestion were examined for binding to heparin by ACE. Heparin was included in the running buffer, and the migration time of peptide was compared with a control (Fig. 5). The binding interactions were characterized by analysis of changes in peak appearance and migration times. Next, the main heparin-interacting peptide was purified from the SAP digest and subjected to analysis and examined for heparin binding by ACE (Fig. 6). By comparing the analysis profile with that of the control experiment, the changes in the migration of complexing peptides were noted. A concentration-dependent decrease in the mobility of the peptide was observed with increasing concentration of heparin in the electrophoresis buffer. No changes in peak shape or size were detected. As expected, the equilibrium shifts toward longer times spent in the complexed form when more heparin is present. Additionally, the heparin-binding sequence in the peptide fragment was characterized and identified by mass spectrometry and amino acid sequencing. This report showed the potential use of the ACE technique for directly mapping binding sites in protein digest.

2. Example 2: Using ACE to Separate Heparin-Binding Peptides

Immobilizing heparin and heparan sulfate onto fused-silica capillaries using biotin–neutravidin conjugation has been applied to ACE analysis (73). These capillaries exhibit markedly reduced electroosmotic flow and were able to distinguish heparin-binding peptides that differed only in the stereochemistry or type of amino acid residue. No resolution of these peptides was observed if heparin or heparan sulfate was included in the buffer phase.

Capillary Modification. The capillary was chemically modified based on the immobilization method used by Cosford and Kuhr (84). The chem-
Fig. 6  ACE analysis of a purified heparin-binding peptide fragment of SAP. Demonstration of interaction with heparin. The peptide fragment was analyzed at 20 kV (detection at 200 nm) after a 5-s injection in the presence of the indicated concentrations of heparin in the electrophoresis buffer (0.1 M phosphate, pH 7.5). (From Ref. 71.)

Chemistry of immobilization of the heparin and heparan sulfate onto the surface of the capillary is illustrated in Figure 7. Briefly, the capillary was treated with 3-aminopropyl triethoxysilane (APTES), succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin), and neurtavidin, respectively. Finally, biotinylated heparin or heparan sulfate was introduced to the capillary wall as an affinity layer. Heparan sulfate was immobilized to the surface through biotin
Fig. 7  Schematic diagram showing the immobilization of GAG to the capillary surface. (From Ref. 73.)
covalently attached through its primary amino group, whereas heparin was biotinylated through covalent attachment to its carboxyl groups.

Separation of Heparin-Binding Peptides. Two peptides were synthesized based on the heparin-binding domain of acidic fibroblast growth factor (aFGF) having the sequence GLKKNGSCGRPRTHYGCQA, residues 125–144. These two peptides, which differ only in the stereochemistry of a single amino acid (L-proline to D-proline), showed the interaction to both heparin and heparan sulfate. However, a peptide in which L-proline (the native peptide sequence) showed higher affinity for heparan sulfate. The different dissociation ($K_d$) constants for the heparan sulfate interaction with these peptides were observed by injecting a racemic mixture of D- and L-proline peptides onto the heparan sulfate–coated capillary, and the mixture was readily separated, as shown in Figure 8. The separation is based on the different affinities of the peptides to heparan sulfate resulting from the different migration times (25.4 and 25.9 minutes for the D- and L-proline-

![Graph](image)

**Fig. 8** Separation of D- and L-proline aFGF peptides using neutral and heparan sulfate–coated capillaries. A racemic mixture of D- and L-proline-containing peptides (130 μM) were injected (22 nL) and subjected to electrophoresis using 50 mM sodium phosphate buffer, pH 7.4, 20C, and 20 kV. (From Ref. 73.)
containing peptides, respectively). No resolution was observed in control experiments using a normal fused-silica capillary and a neutral hydrophilic polymer–coated capillary.

This report also showed a markedly improved resolution in the separation of arginine (R₃W) and lysine (K₃W) basic polypeptides on a heparan sulfate–coated capillary, compared with a neutral-coated capillary. Arginine (R₃W) and lysine (K₃W) polypeptides, while having an identical charge, differ sufficiently in their properties to be partially separated on the neutral-coated capillary (Fig. 9). A heparan sulfate–coated capillary increases the migration times of both peptides. In addition, the separation of these peptides was significantly enhanced due to the different affinities for heparan sulfate of each peptide (Fig. 9). Arginine-rich peptides are known to bind heparin and heparan sulfate with several-fold higher affinity than do lysine-rich peptides (4).

![Graph](image)

**Fig. 9** Separation of K₃W and R₃W peptides using neutral and heparan sulfate–coated capillaries. A mixture of K₃W and R₃W peptides (140 and 400 µM, respectively) were injected (22 nL) onto the capillaries, and electrophoresis was performed as described in Figure 8 legend. (From Ref. 73.)
3. Example 3: Using ACE to Study the Affinity Interaction of Heparin with the Serine Protease Inhibitors

Based on the mixed-phase method, ACE is introduced for studying the interaction of heparin with the serine protease inhibitors, antithrombin III (ATIII) and secretory leukocyte proteinase inhibitor (SLPI) (85). An etched capillary, to which heparin has been covalently immobilized, was used in this study. This modified capillary both afforded an improvement in the separation of heparin-binding proteins and required a lower quantity of loaded protein.

Capillary Modification. The capillary was etched with a fluoride compound at high temperature to prepare a whisker column with a 1000-fold increased inner surface area as compared to an unmodified capillary (86). This results in almost the same specific surface area as found in capillary packed with macroporous silica beads. Next, the heparin was covalently immobilized on the surface of an etched capillary through a spacer using silane chemistry.

Separation of Serine Protease Inhibitors. As a control experiment, ATIII was injected into an unmodified capillary in the presence of low-molecular-weight heparin. No change in the migration of ATIII under electrophoresis was observed. The interaction between the proteins with heparin was studied by performing ACE on ATIII, SLPI (heparin-binding proteins), and bovine serum albumin (BSA) (noninteracting protein) using heparinized capillary. The proteins were bound to heparinized capillary, washed with buffer, eluted with sodium chloride, and detected by absorbance. Noninteracting BSA eluted first in buffer, while SLPI and ATIII, having affinity for heparin, were eluted only when the capillary was washed with buffer containing 1 M sodium chloride (Fig. 10). This study demonstrates a new approach to measuring the heparin interaction of both SLPI and ATIII using ACE.

A detailed examination of the affinity of SLPI for the heparinized capillary was next made using a stepwise elution (from 0.1 to 0.9 M NaCl) (Fig. 11). SLPI eluted from the capillary with 0.2 M NaCl. This agreed well with results obtained by traditional affinity chromatography on a heparin-Sepharose matrix. The ACE method has the unique advantages over traditional affinity chromatography in that it requires much smaller quantities of protein and afforded better separation profiles.

4. Example 4: Using ACE to Observe the Complexation Behavior of Amylodextrin Oligomers and Selected Pharmaceuticals

Amylose and amylodextrins have been used in the food and pharmaceutical industries as excipients. Recently, they have been explored as chiral selec-
Fig. 10  ACE using an etched capillary with heparin bound. (a) SLPI concentration, 10 mg/mL. ACE condition: etched capillary, 75-μm ID × 55 cm (47 cm from injection to detection window), heparin bound via silane spacer. Injection mode: gravity, height 55 cm, time 15 s. Washing and elution mode: pressure injection, 2 psi, 300 s. Buffer A, 25 mM sodium phosphate, pH 7.4; buffer B, buffer and 1.0 M NaCl. Detection wavelength, 220 nm. (b) ATIII concentration, 4.5 mg/mL. (c) Bovine serum albumin, 0.3 mg/mL. (From Ref. 85.)
Fig. 11  ACE step elution of SLPI by various concentrations of NaCl containing buffer from heparin-bound etched capillary. NaCl concentration in elution buffer: see Fig. 10 for conditions. (From Ref. 85.)

tors. A fluorescent-labeling approach was applied to this interaction study between amylopectins and four different pharmaceuticals (ibuprofen, ketoprofen, furosemide, and warfarin) (75). Amylopectin oligomers were fluorescently derivatized. The complexation behavior was predicted based on observed changes in the migration times and peak shapes of the amylopectin solutes obtained under electrophoresis in the presence and absence of the binding partner. Further, $^{13}$C NMR measurements were performed in support of the ACE complexation studies.

The selectivity of complexation was the subject of this study. The minimum size of sugar oligomer chain able to form the complex was explained. As a model experiment, the interaction study between amylopectins and (S)-(+) ibuprofen was performed. The migration profiles of amylopectins with and without ibuprofen ligands in the buffer clearly showed differences. These results suggested a size selectivity for the formation of oligosaccharide drug complex (Fig. 12). No changes in the retention time and peak shape of dextrin oligomers was observed when the degree of polymerization of this oligosaccharide less than 10, indicative of no interaction taking place between these small amylopectins and ibuprofen. Interestingly, the decasaccharide (DP = 10) peak was visibly sharpened. This suggested a strong interaction between the decasaccharide and the drug li-
Fig. 12  Electropherograms of amylodextrins with (A) and without (B) (S)-(+) ibuprofen. Conditions: 46- and 12-cm coated capillaries, 50-μm i.d., 360-μm o.d.; 40 mM acetate-Tris buffer, pH = 5.0, ionic strength 18.3 mM (adjusted by NaCl); LIF detection at 488 nm/514 nm; applied voltage, 25 kV; current: 20 μA. (From Ref. 75.)
The sharp peak associated with the decasaccharide drug complex also migrated faster than the decasaccharide in the noncomplexed form, suggesting conformational change due to a tight interaction and an enhanced charge-to-mass ratio of this complex.

This study also suggests that molecular size and structure play a role in this interaction. The binding behaviors of dextrin oligomers for four different pharmaceuticals (ibuprofen, ketoprofen, furosemide, and warfarin) were observed under the same experimental conditions. Ibuprofen and ketoprofen, two compounds that are similar in chemical structure and pharmaceutical use, showed obvious differences in interaction patterns (Fig. 13A and B). Ketoprofen, having an extra aromatic ring, required an octasaccharide (DP = 8) for binding, whereas ibuprofen required a heptasac-
charide (DP = 7) for its interaction. Furosemide and warfarin required longer oligosaccharide chains for complexation, consistent with their larger molecular sizes (Fig. 13C and D).

Several factors that might influence the actual interaction were also examined. Experimental conditions such as pH, ionic strength, and the nature and concentration of organic additives significantly affected the formation of the complex.

VIII. CONCLUSIONS AND FUTURE DIRECTIONS

Heparin is the most commonly used pharmaceutical polysaccharide, having been used for over 60 years as an anticoagulant. Numerous proteins of physiologic and pathologic importance have been found to interact with heparin, offering a large number of potential new therapeutic applications for heparin. A major limitation in utilizing heparin in new ways is that its anticoagulant properties can result in hemorrhagic complications. The introduction of LMW heparins, heparin oligosaccharides, and synthetic analogues of reduced anticoagulant activity has been used to enhance heparin's specificity, required for new applications in the treatment of cancer, viral and bacteria infection, Alzheimer's disease, and transplant rejection. To substitute for heparin in these new applications, an analogue should ideally bind only those proteins that regulate the desired activity. Interaction studies have been performed on heparin and its analogues using a variety of methods. The ACE technique has been the subject of much attention because of its high resolution, high selectivity, and high throughput and the extremely small amounts of sample required. And ACE offers advantages to pharmaceutical and medical researchers in their study of drug interaction with polysaccharides used as excipients (see Chapter 4).

Although the ACE method offers a number of advantages, several important problems still need to be addressed. A major issue is the concentration detection limit. The development of more sensitive detection methods, such as mass detector and LIF detection, should be very helpful in overcoming this marked disadvantage. The combination of CE, fluorescent labeling, and LIF detection might provide an efficient way to quantify the amount of unlabeled molecules in the form of a fluorescent complex. However, a marked disadvantage of LIF is that labeling of the analytes is usually required, which might change the interaction behavior of the solutes under investigation. An alternative detection approach, mass spectrometric (MS) detection, looks very promising. The combination of the high selectivity of ACE and the structural identification of the MS makes ACE-MS a powerful
technique (see Chapter 13). Unfortunately, this combination technique is expensive and still not routinely available.

The interaction of the analytes with the capillary wall is another problem that prevents the application of ACE in many cases. Coated capillaries might represent one approach to overcoming this problem. Recent efforts have focused on developing simple, reproducible, and chemically stable coating procedures. There are still many challenges to be solved in developing such coated capillaries.

Important future progress in sensitive detection strategies and the emergence of adequate coating technologies should increase the application of ACE in the study of polysaccharide–protein interaction. This will greatly increase our fundamental understanding of the biology, chemistry, and physics of these interactions. Practical application of ACE is also expected in the fields of high-throughput screening and combinatorial chemistry. Finally, this approach will also be extremely useful for clinical chemistry and diagnostics.

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