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Recent advances in biotechnology for heparin and heparan sulfate analysis

Meng Qiao^{a,#}, Lei Lin^{b,#}, Ke Xia^c, Jun Li^d, Xing Zhang^{a,*}, Robert J. Linhardt^{c,e,*}

^aSchool of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Wenyuan Road 1, Nanjing 210023, China

^bSchool of Environment, Nanjing Normal University, Wenyuan Road 1, Nanjing 210023, China

^cDepartment of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy New York, 12180, USA

^dCAS Key Laboratory of Tropical Marine Bio-Resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

^eDepartment of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy New York, 12180, USA

Abstract

Heparan sulfate (HS) is a class of linear, sulfated, anionic polysaccharides, called glycosaminoglycans (GAGs), which present on the mammalian cell surfaces and extracellular matrix. HS GAGs display a wide range of critical biological functions, particularly in cell signaling. HS is composed of repeating units of 1→4 glucosidically linked uronic acid and glucosamine residues. Heparin, a pharmacologically important version of HS, having higher sulfation and a higher content of iduronic acid than HS, is a widely used clinical anticoagulant. However, due to their heterogeneity and complex structure, HS and heparin are very challenging to analyze, limiting biological studies and even resulting in safety concerns in their therapeutic application. Therefore, reliable methods of structural analysis of HS and heparin are critically needed. In addition to the structural analysis of heparin, its concentration in blood needs to be closely monitored to avoid complications such as thrombocytopenia or hemorrhage caused by heparin overdose. This review summarizes the progress in biotechnological approaches in the structural characterization of HS and heparin over the past decade and includes the development of the ultrasensitive approaches for detection and measurement in biological samples.

Graphical Abstract

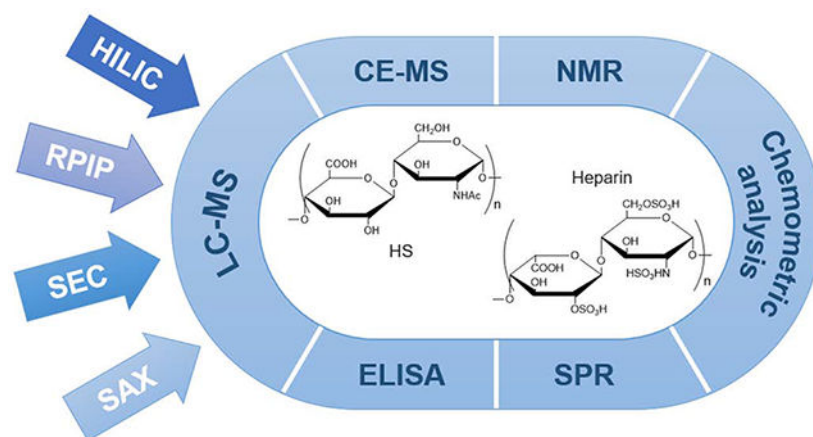
*To whom correspondence should be addressed: XZ zhangxing@njnu.edu.cn and RJL linhar@rpi.edu.

#M.Q. and L.L. contributed equally to this work.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

Heparan sulfate; heparin; structure analysis; ultrasensitive analysis

1. Introduction

Heparan sulfate (HS) is a glycosaminoglycan (GAG), a linear, sulfated, polydisperse, structurally complex hetero-co-polysaccharide [1,2]. HS is comprised of a disaccharide repeating unit consisting of β -D-glucuronic acid (GlcA) or α -L-iduronic acid (IdoA) and β -D-glucosamine (GlcN) linked by (1 \rightarrow 4) glycoside bonds (Figure 1). Modifications of disaccharide units, including the presence of *N*-acetyl or *N*-sulfo substituents on the GlcN (GlcNAc or GlcNS), *O*-sulfo groups at the 6-*O*-and/or 3-*O*-positions of GlcN, and 2-*O*-sulfo substituents on the IdoA positions, making HS diverse structurally (>32 possible units) [3]. HS is widely distributed on animal cell surfaces and in the extracellular matrix (ECM) [2]. There HS binds to many ECM proteins (*i.e.*, growth factors, chemokines, adhesion proteins, etc.) [4,5], allowing these to act as signaling molecules, playing an important role in cell adhesion, migration, differentiation, signaling and angiogenesis [6,7]. Most importantly, HS has physiological and pharmacological impact in blood coagulation, embryonic development, inflammatory response, and bacteria/viral infections [3,8].

Heparin is a GAG sharing the same biosynthetic pathway as HS but with a higher level of sulfation and prominent repeating trisulfated disaccharides, GlcNS6S(1 \rightarrow 4)IdoA2S (Figure 1) [9]. For more than 100 years, heparin has been clinically used as a drug for the treatment of thrombotic disorders [10]. Heparin binds to antithrombin III (AT), an inhibitor of serine proteases, factor Xa and thrombin, resulting a conformational change amplifying the inhibitory activity of AT [3,11]. There are three major forms: the *intravenous* drug, unfractionated heparin (UFH, MW_{avg} 16,000 Da); several types of *subcutaneous* low molecular weight heparins (LMWH, MW_{avg} 3500–6000 Da; and *subcutaneous* ultralow molecular weight heparins (ULMWH, MW < 2000 Da) [3,9]. There is an antidote, cationic peptide drug, protamine, which can reverse the effect of UFH. Life-threatening complications, such as heparin-induced thrombocytopenia (HIT) can occur particularly with UFH [12]. For the past 30 years LMWH such as enoxaparin (Figure 1, 4), generated from

UFH by chemical β -elimination, have been widely used because of their longer half-life and *subcutaneous* bioavailability [13] LMWH binds to AT and acts only on FXa without inhibiting thrombin, facilitating a more subtle regulation of coagulation and an improved therapeutic index [14]. A synthetic ULMWH, fondaparinux, which is the first chemically synthetic antithrombotic pentasaccharide [15], is infrequently used due to its high cost (Figure 1, 3).

Recently, biotechnological versions of UFH, LMWH and ULMWH have reached the stage of commercial development [16,17,18]. These products rely on chemoenzymatic synthesis making their preparation more cost competitive than products like fondaparinux, which relies on chemical synthesis. New analytical approaches that are information rich, sensitive, robust and reliable are required in the development of this new generation of biotechnological heparin and HS products. This review focuses on the analytical methods developed over the past decade.

2. Why is analysis of heparin and heparan sulfate important?

The UFH and LMWH currently in clinical use are extracted from animal tissues, such as porcine, bovine intestines and bovine lungs [19]. As a result, these products have purity and homogeneity issues and a variable based on animal species, age, sex, health, animal feed types and other environmental factors [20]. More importantly, there is a risk of viral or prion infection, which poses a serious threat to the safety of these drugs. A global contamination of heparin broke out in 2007, causing over 200 deaths in the US [21]. This crisis resulted from the adulteration of heparin with a toxic and inexpensive oversulfated chondroitin sulfate. Therefore, reliable structural analysis techniques for animal sourced heparin are highly desirable. Biotechnologically prepared heparins and HS offer different but equally challenging analytical objectives. Subtle differences in the structures or process artifacts require new information rich in highly sensitive analytical methods. For instance, the detection of low level process impurities or relatively subtle structural features, such as sugar epimers or sulfo group position or differences in linkage position and anomeric configuration, require more advanced analytical methods. In addition, sensitive, reliable and robust methods to determine heparin levels within biological fluids and tissues are needed to avoid complications such as hemorrhage induced by heparin overdose. Thus, this review will summarize the progress of the structure characterization of heparin/HS in the past decade, and discuss the development of new ultrasensitive approaches to monitor heparin and HS-based therapeutics.

3. Techniques for structure characterization/quantification of heparin/HS

Characterization strategies for heparin/HS, particularly LMWHs, can be generally summarized into two approaches, top-down and bottom-up analysis, and these are based on the Food and Drug Administration (FDA) requirements (Figure 2) [22]. Similar to proteomic strategies, top-down analysis analyzes samples without any chemical or enzymatic pretreatment and can rely on simple methods such as size exclusion chromatography (SEC) to measure the molecular weight, or more complicated methods such as liquid chromatography-mass spectrometry (LC-MS) to obtain fingerprint mapping of intact chains,

MS/MS to sequence chains or nuclear magnetic resonance (NMR) to analyze intact chains [23]. Bottom-up strategies rely on the controlled partial depolymerization of polysaccharide chains to afford oligosaccharides for analysis by high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and LC-MS disaccharide compositional analysis or oligosaccharide sequence analysis [24]. The controlled depolymerization of a heparin/HS can rely on either random or semi-random chemical methods or somewhat more controlled enzymatic methods. Heparinases, either lyases [25] or hydrolases [26] provide a mild method for the controlled enzymatic depolymerization of heparin/HS. The individual methods applied in both top-down and bottom-up will be discussed in detail.

3.1. Liquid chromatography-MS

Liquid chromatography (LC) is a separation and analysis tool relying on a liquid as mobile phase and a stationary solid phase. High-performance liquid chromatography (HPLC) provides faster separation and higher resolution method than LC and has been widely used in the analysis of GAG disaccharides and oligosaccharides [27,28]. Using different mobile and solid phases, based on different separation principles, HPLC can facilitate the separation and analysis of HS/heparin mixtures and provide diverse structural information. HPLC separations of HS/heparin include size exclusion chromatography (SEC) strong anion exchange (SAX)-HPLC, reversed-phase (RP)-HPLC, reversed-phase ion-pairing (RPIP)-HPLC, and hydrophilic interaction chromatography (HILIC) [25,29]. Ultraperformance liquid chromatography (UPLC), microflow, nanoflow, capillary HPLC and UPLC has also been applied [26]. While LC can be used with a variety of detections such as ultraviolet fluorescence and refractive index, it has most recently been used in conjunction with mass spectrometry (MS), providing high-resolution spectra in both top-down and bottom-up analysis affording a stable and fast continuous analysis platform [22,30].

3.1.1. Top-down analysis—Top-down NMR and LC-MS & MS/MS analysis carefully examines the intact HS or heparin chains and can provide excellent results in the analysis of the number and types of sulfo groups, and the types of uronic acid and glucosamine residues present in HS/heparin oligosaccharides and LMWHs [30]. Top-down analysis LC-MS & MS/MS has still not yet been successfully applied to intact chains of UFH because of its relatively high molecular mass (>10,000) and net charge (>-40). There are challenges for using MS analysis with a top-down strategy, in particular, the loss of sulfo groups, and production of multiple adducts. While Fourier transform (FT)-ion cyclotron resonance (ICR) MS offers a high-resolution platform, but the availability of such instrumentation has been problematic. Matrix assisted laser induced ionization (MALDI) MS has limitations in the analysis of highly charged heparin oligosaccharides of large size [31]. The widespread availability of Orbitrap instruments has alleviated this limitation to some extent [32]. RPIP-LC, the first LC-MS method for the analysis of LMWH had problems of instrument contamination with non-volatile ion-pairing reagents, and relatively low resolution and sensitivity [33]. HILIC is a separation methods based on the disparity between the overall polarity of polysaccharides or oligosaccharides, leading to differences in their interaction with the solid phase support [34]. A relatively high-throughput online analytical platform, HILIC LC-FT-MS with Orbitrap detection resulted in relatively high-resolution, excellent separation efficiency, with good reproducibility and stability (Figure 3). The separation

relied on a Luna HILIC column, which replaced the standard amine with a cross-linked diol solid-phase support, to improve the resolution of complex LMWH mixtures for MS detection. Bioinformatics software (GlycReSoft) was then used to quickly extract analysis data. This rapid, robust method requires no labeling and can be used to compare different commercial LMWHs, analyzing ~90% of the chains, up to dp18 including minor components [35]. Moreover, small amounts of process byproducts, such as chains with 1,6-anhydro reducing ends could be detected and use to compare generic versions to the innovator drug, Lovenox[®]. However, HILIC-LC affords lower resolution than RPIP-HPLC, resulting in challenges for this technology to achieve 100% coverage of chains within LMWH. These challenges might one day be overcome to allow the separation of all of the minor components in LMWH through the use of improved HILIC-LC, and higher resolution MS instruments [35].

3.1.2. Bottom-up analysis—A top-down strategy is not currently applicable to the analysis of full chain length HS or UFH polysaccharides. Thus, a bottom-up strategy, with high reproducibility and accuracy, is widely used to infer polysaccharide structure by analyzing disaccharides or oligosaccharides obtained through controlled depolymerization of the intact polysaccharides using enzymatic or chemical methods [30].

3.1.2.1. Hydrophilic interaction chromatography-MS: A HILIC-HPLC-electrospray ionization (ESI)-Fourier transform (FT)-MS platform was established for the sensitive analysis of all chemically sulfonated GAG-based contaminants such as the oversulfated chondroitin sulfate that played a role in the 2007 heparin contamination crisis (Figure 4A) [36]. This semi-synthetic GAG could not be enzymatically depolymerized, so that reactive oxygen species (ROS) were used to prepare oligosaccharides in a highly reproducible and relatively nonselective way, while retaining their primary molecular structure. This method could successfully detect oversulfated chondroitin sulfate and oversulfated heparan sulfate in the presence of heparin. These, and other potential adulterants, such as *N*-deacetylated/*N*-sulfonated oversulfated chondroitin sulfate, can be detected even at low levels in heparin active pharmaceutical ingredient (API).

In other studies, HILIC-MS was utilized as a high resolution and fast method to analyze oligosaccharides prepared from depolymerization of heparin using heparin lyase II (Figure 4B). Following heparin lyase II depolymerization of enoxaparin it was possible to quantitatively detect four major 3-*O*-sulfo group-containing tetrasaccharides (remnants of the AT-binding site), oligosaccharides with 1,6-anhydro-amino residues (process artifacts in enoxaparin preparation) and three linkage region oligosaccharides (where heparin attached to its core protein). This analysis method allows the comparison of the reducing end groups of different commercial LMWHs that cannot easily be achieved using a top-down strategy. This platform has the advantages of fast separation speed (about 7 min), wide dynamic range (>5000) and high sensitivity (detecting 0.01% components), and provides a relatively high throughput and reliable method to control commercial LMWH products [37].

However, HILIC separation still has major limitations in separating isomeric compositions and disaccharides with similar overall polarity having different sulfation patterns. New high-

resolution HPLC separation methods compatible with MS detection are critically needed to fully analyze HS/heparin polysaccharides and oligosaccharides [27].

3.1.2.2. RPIP-MS: RPIP involves the interaction between a hydrophobic column (*e.g.*, a C18 column) and analyte ion-paired with lipophilic ion-pairing reagents, such as hexylamine (HXA), tributylamine (TrBA), trimethylamine (TEA), and tetrabutylamine (TBA) [38,39]. A RPIP microflow HPLC-ESI-MS approach has been used to analyze most HS/heparin disaccharides with reliability and high separation efficiency and speed [40]. However, when analyzing biological samples, this method cannot fully resolve the disaccharides because of the impact of impurities such as salts and proteins in the samples. UPLC separation, using the 1.7- μm column under high pressure (up to 10^8 Pa), retains the principle of HPLC [29] but improves separation speed, resolution, sensitivity and peak capacity (Figure 5A) [38]. This RPIP-UPLC-ESI-MS approach was used with a bottom-up strategy relying on enzymatic depolymerization to analyze small amounts of HS/ heparin in urine, cells and tissues samples and for the structural profiling of the pharmaceutical heparins. This approach allows the qualitative and quantitative detection of eight HS/heparin disaccharides, with short equilibration time and high sensitivity [38]. However, RPIP-HPLC with MS detection still has the disadvantages of complicated preparation, difficult removal of ion-pairing reagent from the mobile phase and low compatibility with on-line MS detection [39,41,42].

3.1.2.3. SEC & RPIP-Q/TOF-MS: Different types of HPLC can be used in combination to achieve better separation results[14]. For example, LMWHs contain diverse oligosaccharide chains that cannot be separated using HILIC, IPRP or SAX. By using multiple heart-cutting (MHC) to connect SEC-HPLC and RPIP-HPLC and detecting with quadrupole time-of-flight (Q/TOF) MS it was possible to provide a definitive and systematic analysis of LMWH to obtain detailed structural information (Figure 4C) [43]. The first dimension, SEC, separated the oligosaccharides by molecular size, while the second dimension, RPIP, separated the oligosaccharides on the basis of differences in their charge and polarity. This approach facilitated the analysis of many components having different sizes of similar charge or of the same size but different charge. This relatively high-resolution, stable, easy-to-optimize, high-efficiency, MHC-2D LC-Q/TOF MS approach could detect 84 oligosaccharides in nadroparin and 122 oligosaccharide peaks in enoxaparin. This three-dimensional approach has the potential to control commercial LMWH production and to detect, analyze and sequence large oligosaccharide chains.

3.1.2.4. SEC & SAX-MS/MS: SAX-HPLC is a means of separation relying on the interaction between negatively charged HS/heparin and the positively charged solid state support of an SAX column [44]. This method affords higher resolution than HILIC and can easily separate disaccharides with different sulfate levels, especially gratifyingly in the analysis of disaccharides obtained by the cleavage of heparins and LMWH with lyase [45]. Unfortunately, samples bound to SAX columns typically need to be eluted with MS-unfriendly high-concentration sodium salts. Therefore, when analyzing enoxaparin, this method has often been used in combination with UV detection after separating the disaccharide depolymerization using enzymes. This method is highly accurate and does not

require labeling. However, when envisaging the analysis of intact LMWH, this method can only be used with enoxaparin containing double bond as a chromophore, and is not feasible for the LMWH dalteparin sodium due to the lack of chromophore and the higher molecular weight. However, the higher molecular weight and lack of chromophore in dalteparin sodium limits its application. An offline method, which collects, combines, and desalts analytes separated by HPLC allows their analysis by MS, SAX-HPLC-ESI-MS/MS and is useful for sequencing the short-chain oligosaccharides from the LMWH, dalteparin sodium (Figure 5B and 5C) [27]. The short oligosaccharides obtained after preliminary separation by SEC could be further separated by SAX-HPLC using a high resolution ProPac PA1 SAX column. ESI-MS/MS could then provide a sensitive and reliable approach for the direct sequencing of low-purity heparin drug samples. This indispensable and sensitive approach was used for the sequencing of 18 oligosaccharides (from tetrasaccharide to octasaccharide) providing a basis for exploring the precise composition of LMWH.

3.2. CE-MS

CE is an effective technique for separating molecules based on their shape, size, and charge [46,47]. MS can accurately and quickly identify and analyze molecules by measuring the mass-to-charge ratio of ions [48]. CE-MS is becoming one of the most powerful techniques for the separation and identification of HS/heparin oligosaccharide due to a number of advantages including high sensitivity, high resolution, simple operation, high separation efficiency, and a flexible mode of separation [49,50]. However, in the process, an effective interface is essential for combining CE and MS. Insufficient research and development of CE-MS interfaces over the past decade has hindered the application of CE-MS in heparin/HS analysis. A groundbreaking neoteric electrokinetic pump-based CE-MS interface was used to combine a normal polarity CE separation with a positive-ion electrospray ionization MS platform. This interface resulted in great sensitivity, resolution and repeatability allowing the online analysis of heparin disaccharide mixture and LMWH chains (Figure 6A) [51]. This method creatively introduced an uncoated capillary sheath emitter tip as an interface. The negatively charged sugar molecules separated by CE were positively charged with NH_4^+ in ammonium bicarbonate buffer solution and then sprayed into the mass spectrometer for analysis. Reasonable combination of capillary, emitter, MS, rational constitution and ratio between background electrolyte (BGE) and sheath liquid (SL) reduce the generation of bubbles and ensure stable and steady electrospray at extremely low flow rate (nL/min). This gentle detection environment greatly improves the analysis sensitivity and makes the high-throughput detection of HS oligosaccharides possible. Using CE-MS requires no glycan labeling affording a high signal-to-noise ratio, rapid analysis (< 10 min), on a small amount of sample (picograms) and utilizes little buffer (< 10 microliters).

However, HS is negatively charged and highly sulfated HS chains may undergo desulfation when separated by normal polarity mode CE and analyzing by positive-ion MS. Such decomposition can result reducing analysis accuracy. As an alternative approach, using a protein-coated emitter with positively charged as the interface as well as adjusting the BGE, SL and electrodes allows a reverse polarity CE separation and negative-mode electrospray ionization MS (Figure 6B) [52]. This approach is useful in rapid bottom-up and top-down

analysis of LMWH without analyte decomposition. CE-MS has the merits of low false positive results, simple data processing and is capable of detecting and identifying LMWH produced by different manufacturers.

Although a CE-MS interface offers a rapid analytical method and has been commercialized, the sensitivity and reproducibility of this method needs further improvement. Online reverse-polarity negative-ion mode capillary isoelectric focusing (cIEF)-MS based on an electrokinetically pumped sheath liquid nanospray CE-MS for the separation and detection of HS disaccharides (Figure 6C) [53]. Using a high voltage a pH gradient with low pH at the anode and high pH at the cathode is formed within the capillary. Therefore, an analyte with a low isoelectric point (pI) will flow first out of the anode side of the capillary and spray into the mass spectrometer for detection. This method enables separation of positional isomers of disaccharides with the same number of *O*-sulfo groups and also discriminates between *N*-sulfo groups and *O*-sulfo groups. Unfortunately, this method has some limitations in separating GAG analytes with very similar pI values.

By combing reverse-polarity capillary zone electrophoresis with negative-ion mode mass spectrometry (CZE-MS) an effective and sensitive tool has been developed for the analysis of GAGs mixtures (Figure 6D) [49]. Using a cation coated capillary, structurally similar sulfated GAG oligosaccharides and complex mixtures of the same were successfully separated and analyzed with CZE-MS in a fast and reproducible manner. The analysis method was applied to a mixture of heparin/HS oligosaccharides, varying chain length from trisaccharide to dodecasaccharides resulting in more than 80 molecular compositions being identified by accurate mass measurement.

Since the diameter capillary is very small it is prone to blockage and the capillary length has not been optimized or standardized so that the reproducibility of CE-MS analysis still has certain limitations. In addition, as with most liquid phase analyses, standards are required [49].

3.3. Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is based on the principle that an NMR signal changes base on the position of the detected nucleus within a molecule. NMR widely used in analyzing the molecular structure of HS/heparin (including conformation) and detecting sample content and purity [54,55,56]. Heparin contaminants and impurities are difficult to detect as they differ only in sulfo group position, degree of sulfation, and sugar epimers. Although a database of pure HS/heparin oligosaccharides is currently lacking, NMR is considered an advantageous means of determining the fine structure of heparin. A library consisting of 66 types of HS/heparin oligosaccharides of different sizes, sulfation levels and positions were by a biotechnological chemoenzymatic synthesis (Figure 7A–7C) [57]. The library contained diverse structures, including rare GlcNS3S6S, Glc2S and IdoA residues, which are found in low abundance in HS/heparin natural products but are important for a variety of biological activities. In the NMR analysis of this oligosaccharide library prepared using biotechnology, the unique anomeric protons showed broad peaks in the ^1H NMR spectra and low signal intensity in 2D the spectra. The chemical shifts anomeric protons and carbons of the various uronic acid and glucosamine residues could be

assembled in a table representing a valuable database. Signal strength and chemical shifts of residues such as, IdoA2S, could be significantly changed by adding ethylenediaminetetraacetic acid (EDTA) to remove trace amounts of divalent cations greatly improving the accuracy of NMR detection of HS/heparin oligosaccharides. This systematic NMR study paves the way for the characterization of more complicated HS/heparin polysaccharides (UFH) helping to safeguard the quality of UFH and ensuring the similarity of bioengineered heparin products with the current used animal-derived heparins.

In recent years, 2D NMR has shown unique advantages in the analysis of disaccharides and the quantitative detection of heparin and LMWH [58,59,60], and is capable of the detection of contaminants, such as oversulfated chondroitin sulfate (OSCS) in heparin [61,62], as well as the analysis of danaparoid sodium salt, an anticoagulant drug containing HS, DS and CS [63]. Furthermore, 2D NMR can be combined with SAX-HPLC to expand the range of detectable structural features, and become a powerful tool for distinguishing sources, monitoring manufacture and ensuring safety and quality of commercial prepared heparin [45]. In response to the call of FDA, which suggesting introducing bovine heparins to address the shortage of heparin drugs, a combination of 1D and 2D-HSQC NMR methods have been used to detect the origin or blending of heparins from different animals and tissues (Figure 7D) [64]. In addition to testing the purity and safety of HS/heparin oligosaccharide products, NMR can also be used to control the synthesis of heparin in standard and biotechnological production processes. ^1H NMR was used to quantify heparosan, a precursor of bioengineered heparin, in a fermentation supernatant during its production from *E. coli* K5 and to optimize fermentation parameters and calculate the yield [65]. However the sensitivity of NMR analysis was insufficiently high to directly monitor heparosan, thus, there are still some limitations for the detection of GAGs.

3.4. Chemometric analysis

There are inevitably limitations when using a single analytical method such as NMR, HPLC-MS to analyze HS/heparin. Multiple assays can be used with chemometric analysis to build a more complete analytical method for HS/heparin analysis. ^1H NMR analysis of intact heparin was used to determine structural differences among heparin products, and combined HPLC-MS analysis on disaccharides treated with heparin lyases to analyze disaccharide concentrations and bottom-up HPLC-MS analysis heparin lyase 2- resistant tetrasaccharides to determine differences in the anticoagulant activity on 30 heparin samples from different sources (Figure 8) [66]. Comprehensive analysis non-intersecting clusters obtained by principal component analysis (PCA) and the K-means, obtained from the visual data mining software, established a detailed database for meticulous analysis of heparin structure. This database was useful in analyzing the biological source of heparin and could also detect mixtures of these heparins prohibited by US FDA regulations. It is worth noting that the NMR spectrum, which provides an array of data of great significance as a reference, is particularly important in PCA and has important applications in many aspects, such as the identification of animal and tissue source, the purity and safety of biologically sourced heparins [67,68], the structural characterization of heparin derivatives [69], the identification of contaminants [70], the analysis of anticoagulant activity [71], and the detection of commercial heparin sources [72].

3.5. SPR techniques

Surface plasmon resonance (SPR), analyzes the interaction between putative ligands and receptor molecules (*i.e.*, enzymes, peptides, antibodies, and DNA) immobilized on a thin metal layer (*i.e.*, silver, gold, or aluminum films) by detecting refractive index changes of the medium near the nanometal film based on optical biosensor technology. SPR is a label-free, high sensitivity, high repeatability, real time detection method [73,74]. With over three decades of use, SPR is considered to be one of the most efficient techniques for identifying affinity, specificity, and kinetic parameters between macromolecules (*i.e.*, protein-polysaccharide, receptor-drug, enzyme-substrate, *etc.*) [75]. SPR can be used to detect the interaction of viruses with GAGs to understand viral pathogenesis and develop treatments. SPR technology for the study of Zika virus (ZIKV), recognized in 2016 by the World Health Organization (WHO) to cause severe congenital defects, was used to explain the role of GAGs in placenta and brain host cells for ZIKV entry (Figure 9A) [76]. ZIKV envelope protein (ZIKV E) has a strong affinity for long-chain heparin oligosaccharides (octasaccharide to octadecasaccharides) and heparins with higher sulfate levels. Human placenta chondroitin sulfate (CS) also binds more tightly to ZIKV E than does porcine brain HS, suggesting that placenta CS may be the most likely receptor mediating ZIKV invasion through the placenta. The porcine brain HS was enriched in trisulfated disaccharide presumably resulting in tighter binding ability ZIKV E. The exploration of the interaction of HS and CS with ZIKV E, using SPR, not only lays a foundation for further understanding ZIKV pathogenesis, but also provides a new approach studying the mechanism of viral infection. SPR is also useful for assessing the anticoagulant activity of heparin. Competitive SPR to test the ability of soluble heparin to compete with AT preventing its binding to heparin immobilized on a chip (Figure 9B–9E) [77]. This competitive binding method correlates well with standard bio assay method, and it reduces the required steps, shortens analysis time, and reduces assay costs while maintaining repeatability. However, the current instrument costs for performing SPR are extremely high limiting this method in point-of-care (PoC) assay until miniaturized and inexpensive SPR sensors become available [78].

3.6. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a highly sensitive bioassay method based on antigen-antibody binding that can be used in both qualitative and quantitative determinations [79,80]. ELISA with enzyme signal amplification has been used as a high-throughput platform for the detection of 3-OST sulfation of a bioengineered heparin precursor (a non-anticoagulant heparin missing its 3-*O*-sulfo group made in a biotechnological chemoenzymatic process (Figure 10A) [81]. Biotin-labeled UFH is immobilized on the inner surface of a 96-well plate coated with streptavidin. After the addition of AT and bioengineered heparin precursor for incubation and AT antibody was added, a secondary antibody horseradish peroxidase (HRP)-conjugated IgG, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate were then added for enzymatic signal amplification. The anticoagulant activity of the bioengineered heparin could then be determined by UV measurement to assess the progress of 3-OST catalyzed reaction. After improving detection performances such as shortening time and reducing costs by continuously optimizing the main factors affecting signal response. This sensitive and accurate detection method

provides a new approach for ensuring the consistent biotechnology process for the industrial production of bioengineered heparin.

3.7. Ultrasensitive analysis of heparin and heparan sulfate

During clinical procedures such as cardiovascular surgery and kidney dialysis, heparin levels need to be closely monitored to avoid complications such as hemorrhage induced by heparin overdose and HIT. The therapeutic dosing level of heparin is 2-8 U mL⁻¹ (17-67 mM) during cardiovascular surgery and 0.2-1.2 U mL⁻¹ (1.7-10 mM) in postoperative and long-term care [82]. Thus, simple, real-time continuous measurements of heparin levels in serum during cardiovascular surgery and the postoperative therapy period, is desirable. There is also a need for detection methods to monitor heparin in infusion solutions to avoid human dosing errors, particularly in pediatric patients. Traditional clinical methods for heparin detection rely on bioassays such as activated clotting time (ACT) or activated partial thromboplastin time (aPPT) [83]. These methods are often not reliable and inaccurate are subject to interference and are difficult to perform in clinical settings. Thus, developing new methods for heparin detection, especially the ultrasensitive determination is desirable, not only facilitate clinic studies, but also improve our understanding of the biological roles of heparin involved in the critical biological processes that it regulates. However, ultrasensitive analysis of heparin is very challenging due to the low concentrations present in complex biological fluids (*i.e.*, plasma or serum) as well as the chemical complexity of glycan chains present in UFH and LMWH drugs. Heparin has no natural chromophores or fluorophores, and because of its highly negative charge often shows poor ionization efficiency in MS analysis. Heparins cannot be detected by amplification methods such as those used to detect nucleic acids, and in contrast to proteins, there are few available carbohydrate-specific antibodies.

Polymerase chain reaction (PCR) is a technique capable of amplifying DNA fragments exponentially for ultrasensitive detection [84,85]. HS/heparin detection cannot be achieved signal amplification used to detect DNA. A biotin-labeled oligosaccharide conjugated to DNA “bar code” constructed and glyco-qPCR was explored as an ultrasensitive online detection platform giving higher sensitivity than MS and fluorescence detection (Figure 10B) [86]. Biotinylated CS disaccharide (as a model system) obtained by reductive amination with a DNA “bar code”, and unreacted DNA completely removed. The CS-DNA conjugate was then used as a template for Qpcr. The results showed that the platform could detect CS oligosaccharides at concentrations < 1 zmol. However, this platform can only analyze to detect the total content of a particular GAG. If complex detection, such as composition, is required, glycol-qPCR requires a micro-separation step. When coupled with CE, glyco-qPCR could detect as few as 500 molecules of CS present in Chinese hamster ovary (CHO) cells. These results suggest the possibility of ultrasensitive detection and the single-cell analysis of GAGs. This approach can also be used for the sensitive analysis of GAG-protein interactions providing a reliable basis for exploring the mechanism of GAGs actions and in drug development. Although these experiments only applied glyco-qPCR to the CS GAG it should be possible to apply this ultrasensitive method to the detection of HS/heparin.

This method is not yet sufficiently mature to apply to the analysis of more complex oligosacchene and polysaccharide analytes. The automation and integration of glyco-qPCR for the ultrasensitive analysis of heparin/HS oligosaccharides and polysaccharides should one day be possible. One major challenge is the complete removal of the unreacted DNA as residual DNA can adversely impact on the accuracy and sensitivity of the analysis.

Fluorescence resonance energy transfer (FRET), a technique that utilizes a phenomenon of fluorescence energy transfer that occurs at a suitable distance between the donor and acceptor fluorophores [87,88,89,90], has been applied to the ultrasensitive analysis of HS/heparin [91,92]. An approach for the detection of a chondroitin sulfate disaccharide as a model GAG oligosaccharide was developed based on FRET using a CdSe-ZnS core-shell nanocrystal quantum dot (QD) donor and a Cy5 acceptor [93]. This method allows the ultrasensitive detection of GAGs disaccharides with a sensitivity more than 1,000 times higher than CE-laser-induced fluorescence (LIF) (Figure 11A–11C). In addition, there are currently studies using fluorescent probes to detect heparin in plasma. Wartinger *et al.* using Heparin Red, a commercial fluorescent probe that can oligomerize polyanionic polysaccharides to quench fluorescence, can result in the quantitative detection of non-anticoagulant heparin in human plasma, such as *N*-desulfated-*N*-acetylated heparin and LMWH derivative, tafoxiparin (Figure 11D) [94].

4. Conclusions and perspectives

HS and heparins are important GAGs that are responsible for a range of physiological and pharmacological functions. However, due to the high heterogeneity and complex structures, their analysis is very challenging, limiting their biotechnological synthesis, biological studies, and preventing the adequate addressing of safety concerns. After the heparin contamination crisis of 2007, the US FDA has promoted the diversification of heparin sources such as the reintroduction of bovine heparin or the introduction of bioengineered heparin into the US market. These urgings have stimulated the study of heparin analysis resulting in the steady improvement in this research field. MS is still the most widely used analytical tool particularly when coupled to separation methods, providing high sensitivity, high resolution, and high specificity. In particular, the development of MS and MS/MS in recent years has provided more comprehensive information on the fine structures and domain structures of HS/heparin and has greatly improved detection sensitivity. In addition, combined with stable isotope labeling, LC-MS/MS represents one of the most promising methods to investigate the roles of heparin/HS in metabolic pathways to distinguish exogenously administered stable isotope labeled heparin/HS from their endogenously biosynthesized counterparts [95]. CE-MS has recently gained popularity with the introduction of new CE-MS interfaces due to its short separation time, high efficiency, and the small amounts of sample required. NMR is able to analysis disaccharide, characterize heparin and LMWH and distinguish contaminants present in UFH or LMWH drugs. Despite its relatively low sensitivity, NMR affords high resolution capable of assessing glycosidic bonds, uronic acid epimers and sulfo group substitution. Chemometric analysis can be used to combine a variety of analytical methods and apply statistics and machine learning to provide more comprehensive structural information. SPR uniquely and rapidly analyzes HS/heparin interactions with proteins in real time. The various analytical techniques introduced

in this review can be applied to specific analytical problems or coupled to one another to facilitate the heparin/HS analysis. Reliable ultrasensitive analysis techniques for heparin are also needed clinically to avoid complications such as hemorrhage and HIT.

In the future, the analysis of HS/heparin will continue to develop resulting in even higher sensitivity, higher resolution, shorter analysis times, lower cost, more user-friendliness, higher throughput and improved automation. The development of analytical technology should promote a better understanding of the functions, pharmacodynamic characteristics, and pharmacokinetics of HS/heparin, the analysis of commercial drugs, and the characterization of biotechnological products such as bioengineered heparins and chemoenzymatically synthesized HS/heparin oligosaccharides.

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Highlights

- Heparan sulfates are structurally complex bioactive polysaccharides
- The analysis of complex heterogeneous polysaccharides is difficult
- Mass spectral analysis is a detailed and sensitive method for analysis
- Hyphenated techniques and chemometric analyses are playing a greater role
- Ultrasensitive methods are required for analyzing biological samples

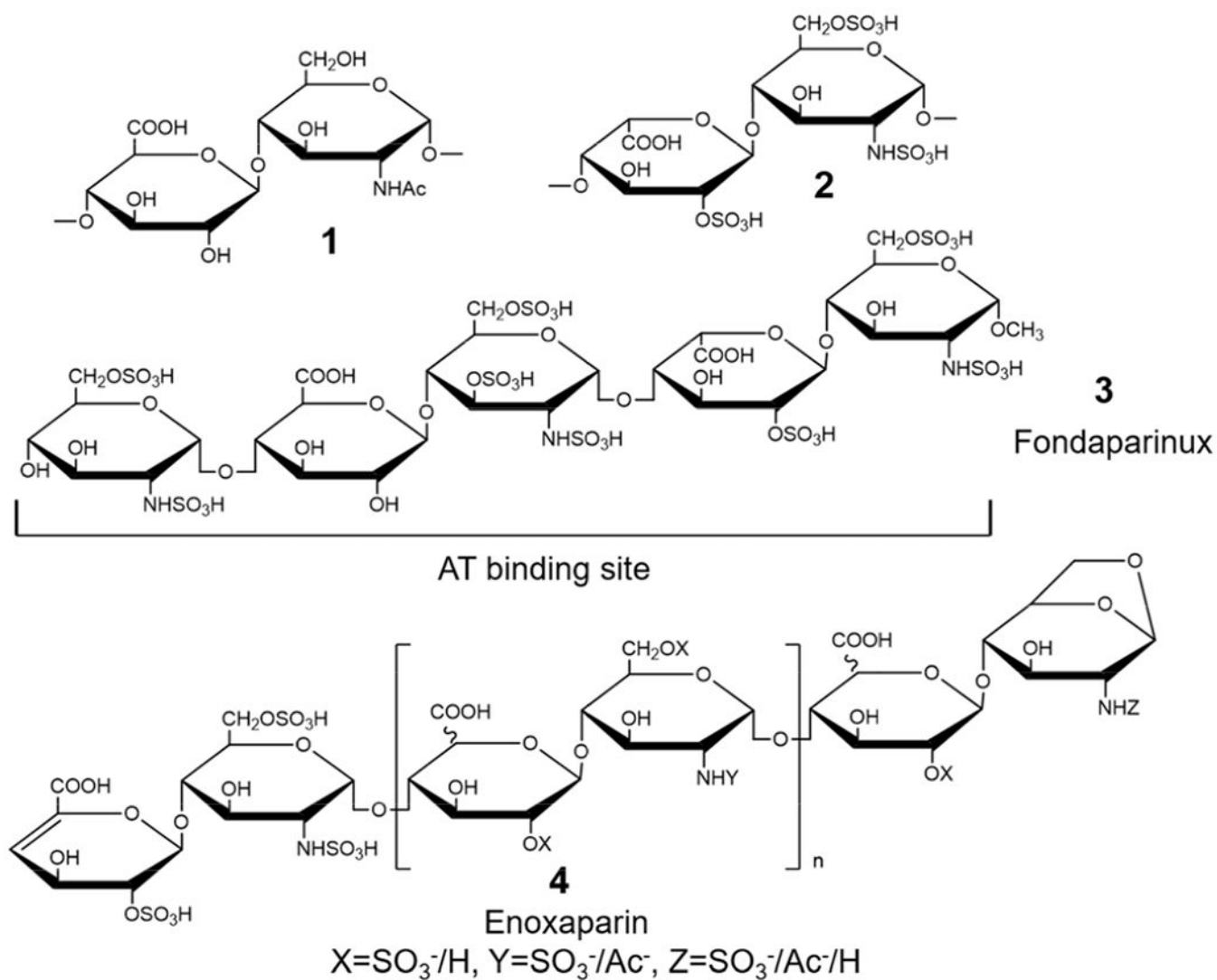


Figure 1.

Structures of HS, heparin and heparin derivatives. The major disaccharide repeating units of HS (primarily **1**) and heparin (primarily **2**) are shown. The structure of an ULMWH, Arixtra® (fondaparinux) **3**, containing a pentasaccharide sequence corresponding to an AT-binding site is shown. The LMWH, Lovenox® (enoxaparin) **4** contains a diverse mixture of chain types but only one type of chain present in enoxaparin, containing a 1,6-anhydro group unique to enoxaparin, is shown.

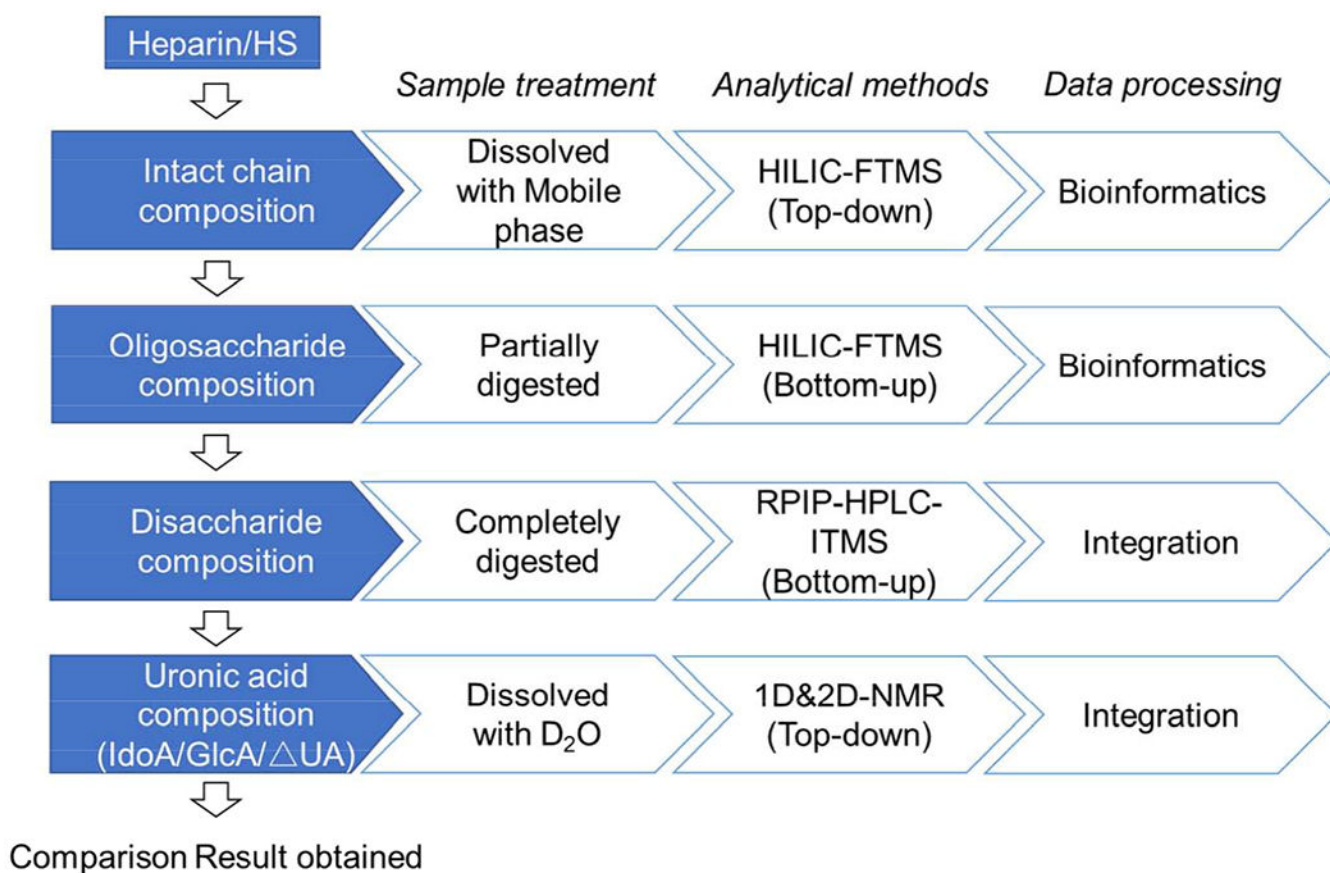


Figure 2. Representative strategies for heparin/HS structural characterization.

Extracted ion chromatography of the major LMWH chains

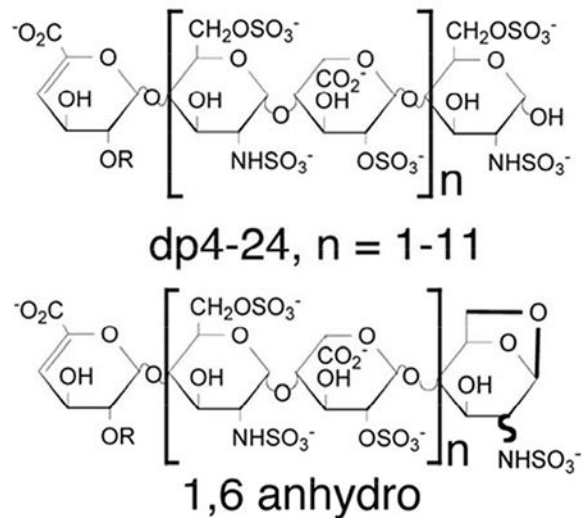
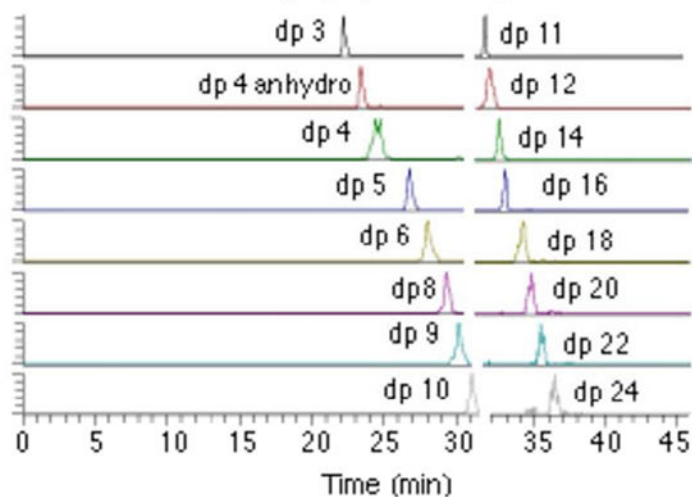


Figure 3. Top-down approach for the direct characterization of low molecular weight heparins using HILIC-LC-FT-MS analysis. Copyright 2012 American Chemical Society.

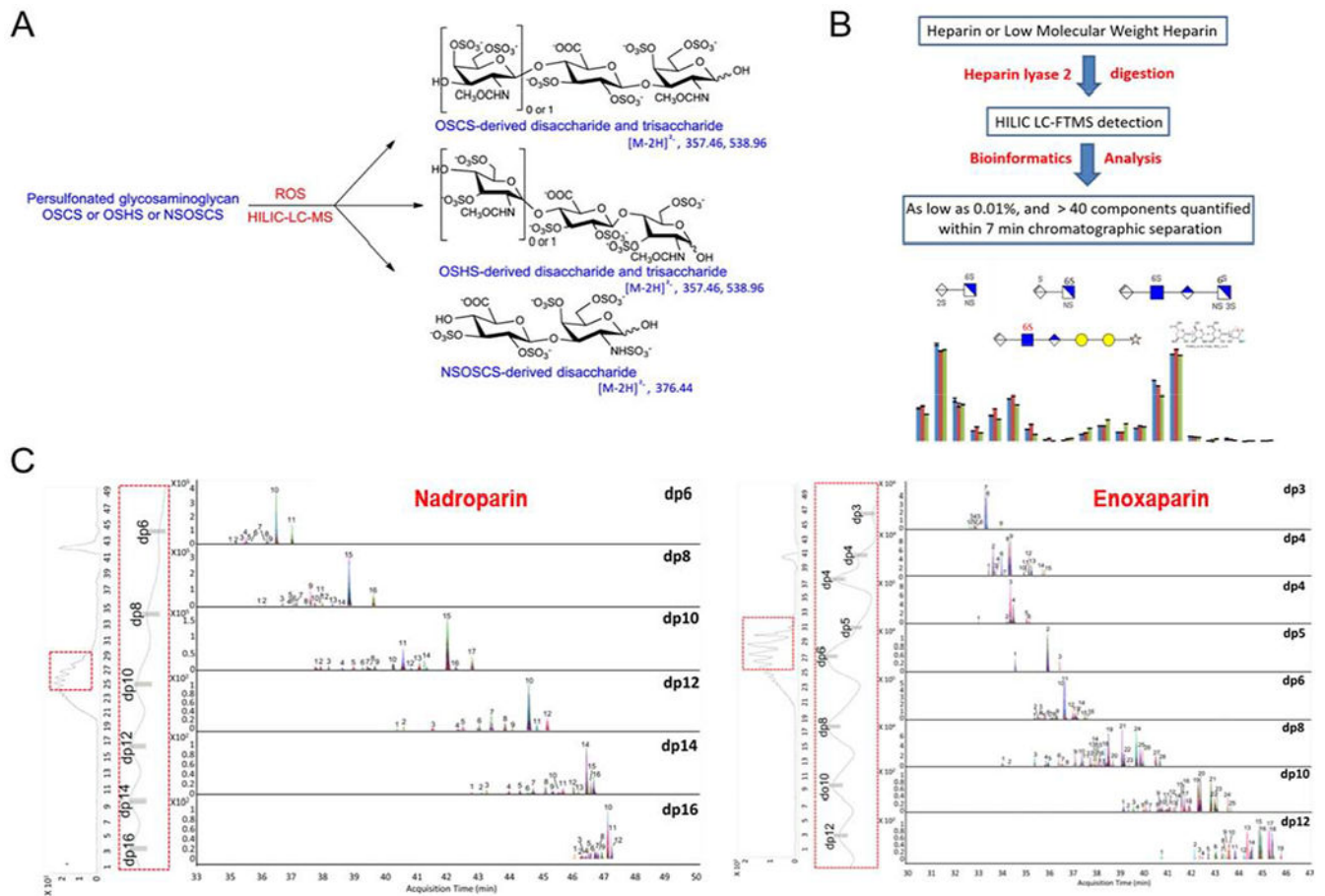
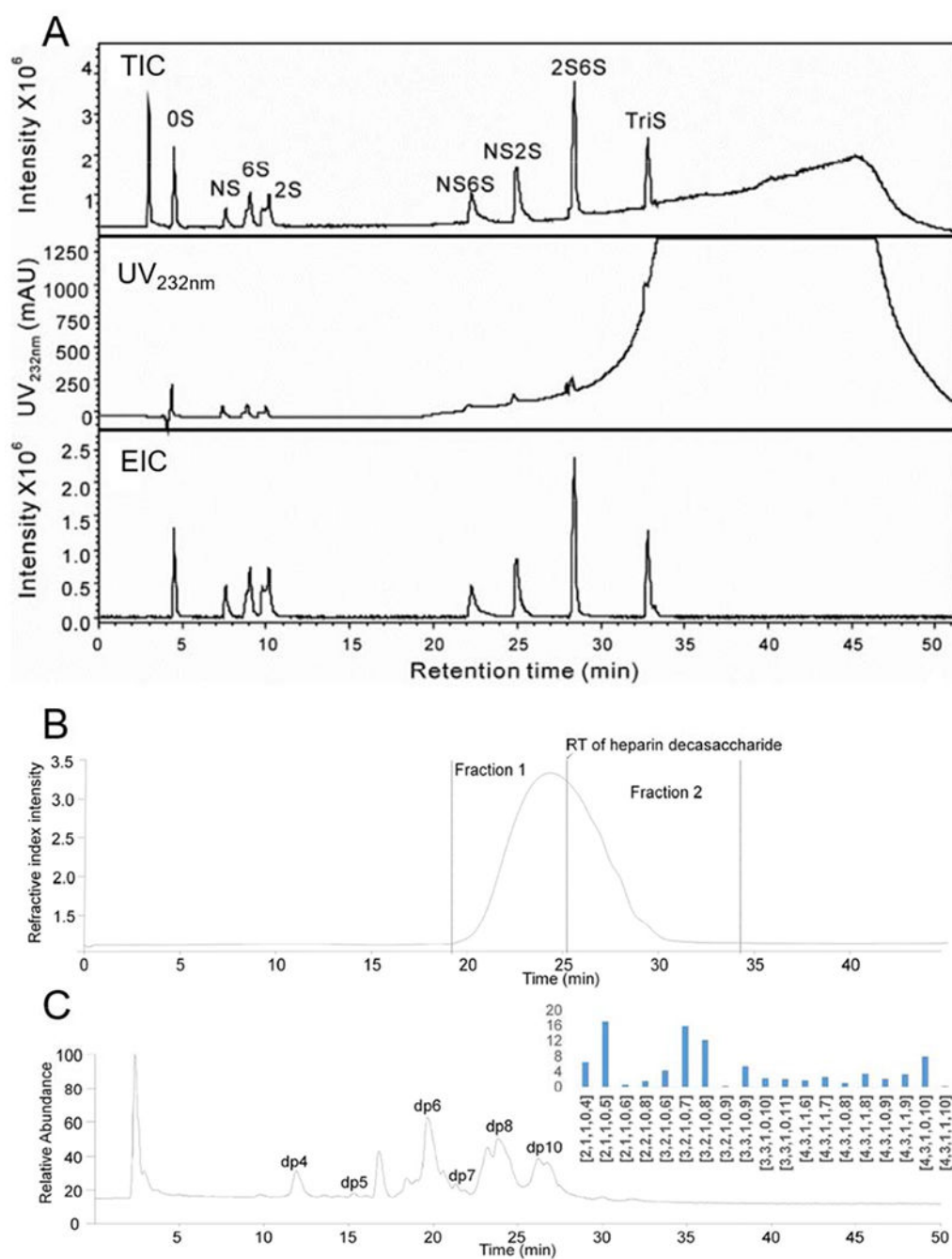


Figure 4. (A) Oxidative depolymerization and HILIC-MS of heparin and oversulfated semi-synthetic GAGs. Copyright 2014 American Chemical Society. (B) Liquid chromatography-Fourier transform mass spectrometry for extensive characterization of low molecular weight heparin. Copyright 2014 American Chemical Society. (C) Profiling analysis of the LMWHs nadroparin and enoxaparin using MHC-2D-LC-Q time-of-flight (TOF)-MS. Copyright 2015 American Chemical Society.

**Figure 5.**

(A) RPIP-UPLC chromatograms of eight heparin/HS-derived disaccharide standards with total ion chromatogram (TIC), UV232 nm, and extracted ion chromatogram (EIC) detection. Copyright 2011 Elsevier. (B) SEC separation of dalteparin; (C) HILIC-ESI-MS of the short dalteparin oligosaccharides collected from SEC and the short oligosaccharide compositions. Copyright 2018 Elsevier.

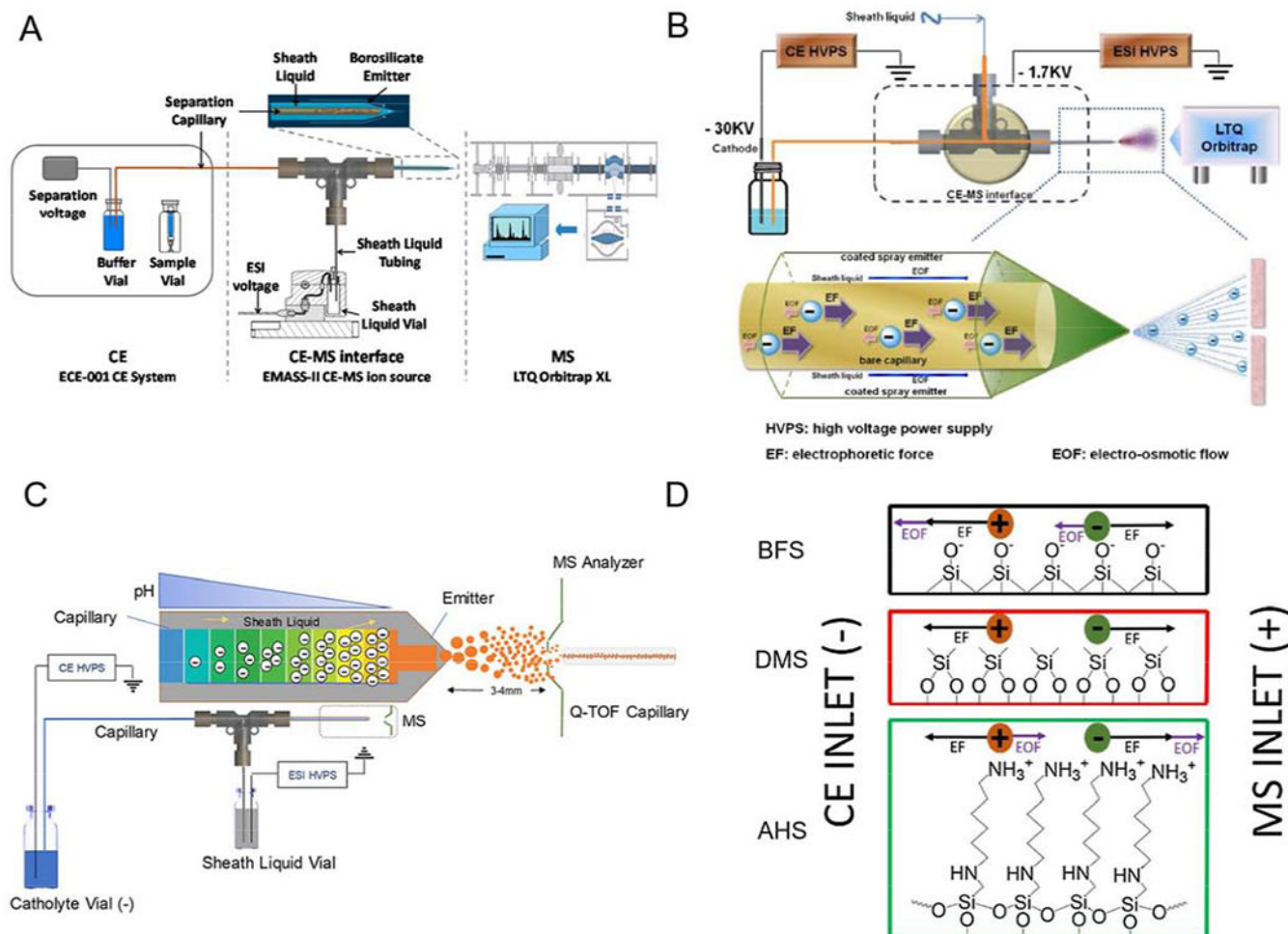


Figure 6. Schematic diagram of CE-MS system under positive-ion mode (A) (Copyright 2016 American Chemical Society.) and negative-ion mode (B) (Copyright 2016 Springer.), respectively. (C) Charge-based separation of acidic oligosaccharides using negative-ion mode capillary isoelectric focusing (cIEF) mass spectrometry. Copyright 2019 American Chemical Society. (D) Diagram depicting the forces of electroosmotic flow and electrophoretic forces that act on analytes during a capillary zone electrophoresis (CZE)-MS experiment with three different inner capillary coatings, BFS (Bare fused silica), DMS (dichlorodimethylsilane) and AHS (N-(6-aminohexyl) aminomethyltriethoxysilane). Copyright 2018 Elsevier.

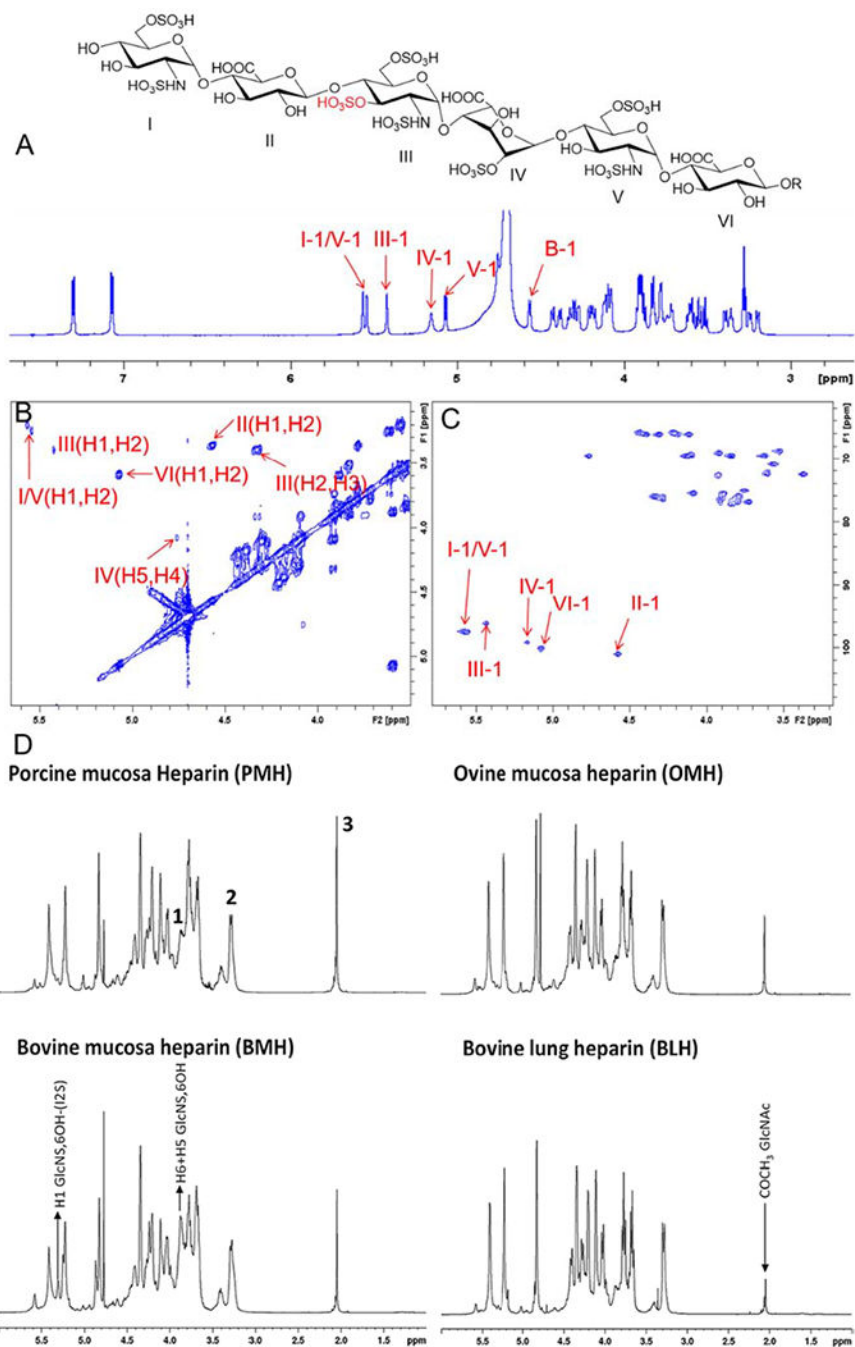


Figure 7.

(A-C) Example of full NMR characterization of a heparin hexasaccharide prepared through chemoenzymatic synthesis. Copyright 2017 The Royal Society of Chemistry. (D) Heparin samples extracted from different species/organ. Signals 1, 2, and 3, corresponding to H5 and H6 of 6-*O*-desulfated glucosamine, H2 of *N*-sulfated glucosamine, and acetyl group of *N*-acetylated glucosamine, respectively, were used as diagnostic peaks for the determination of their origin. Copyright 2019 Frontiers.

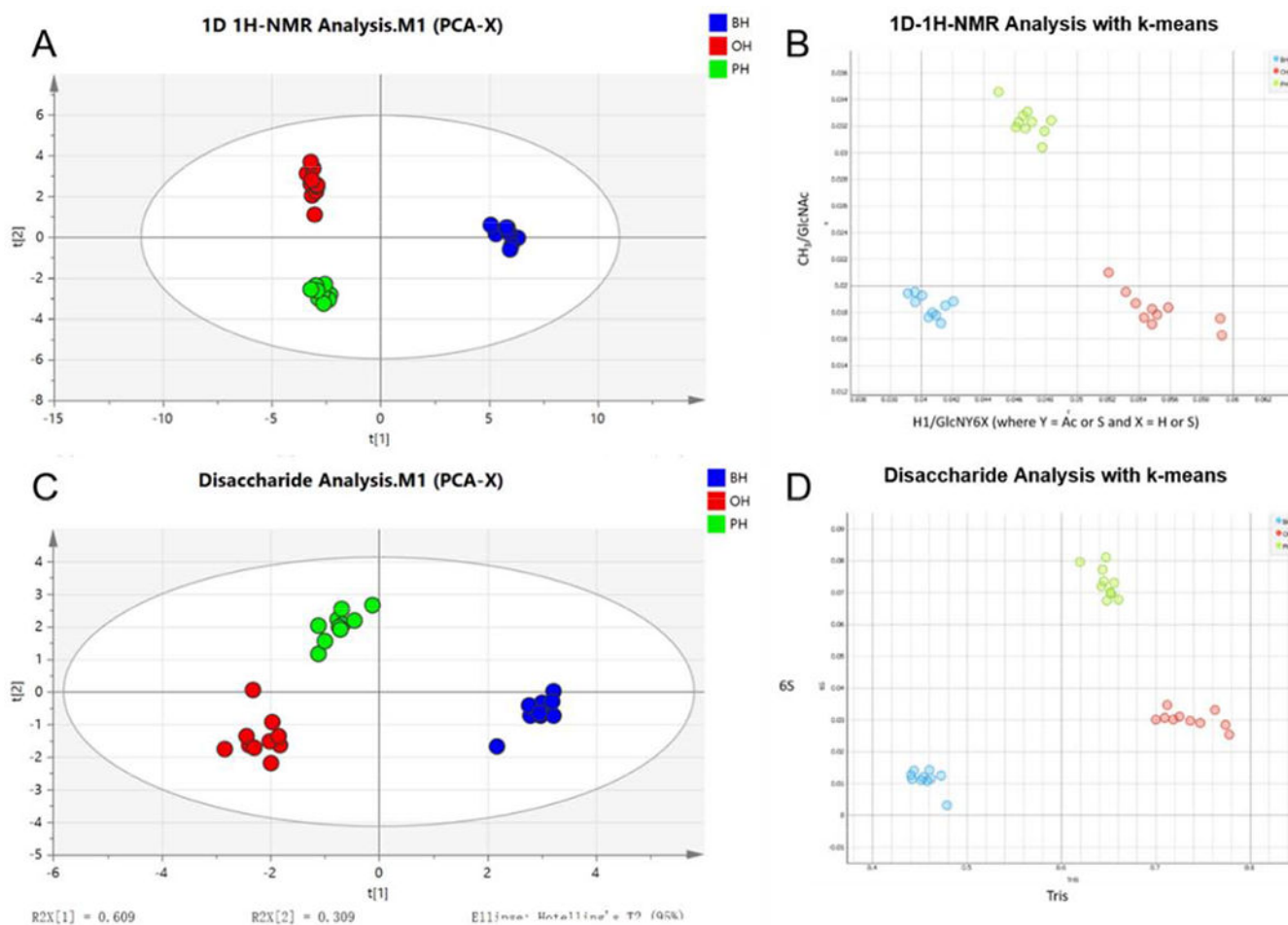


Figure 8. Statistical analysis of bovine, ovine, and porcine UFHs based on ^1H NMR information (A,B) and LC-MS disaccharide analysis (C,D). PCA score plot of two first principle components and K-means clustering analysis of the NMR data and disaccharide data are presented. Copyright 2019 Elsevier.

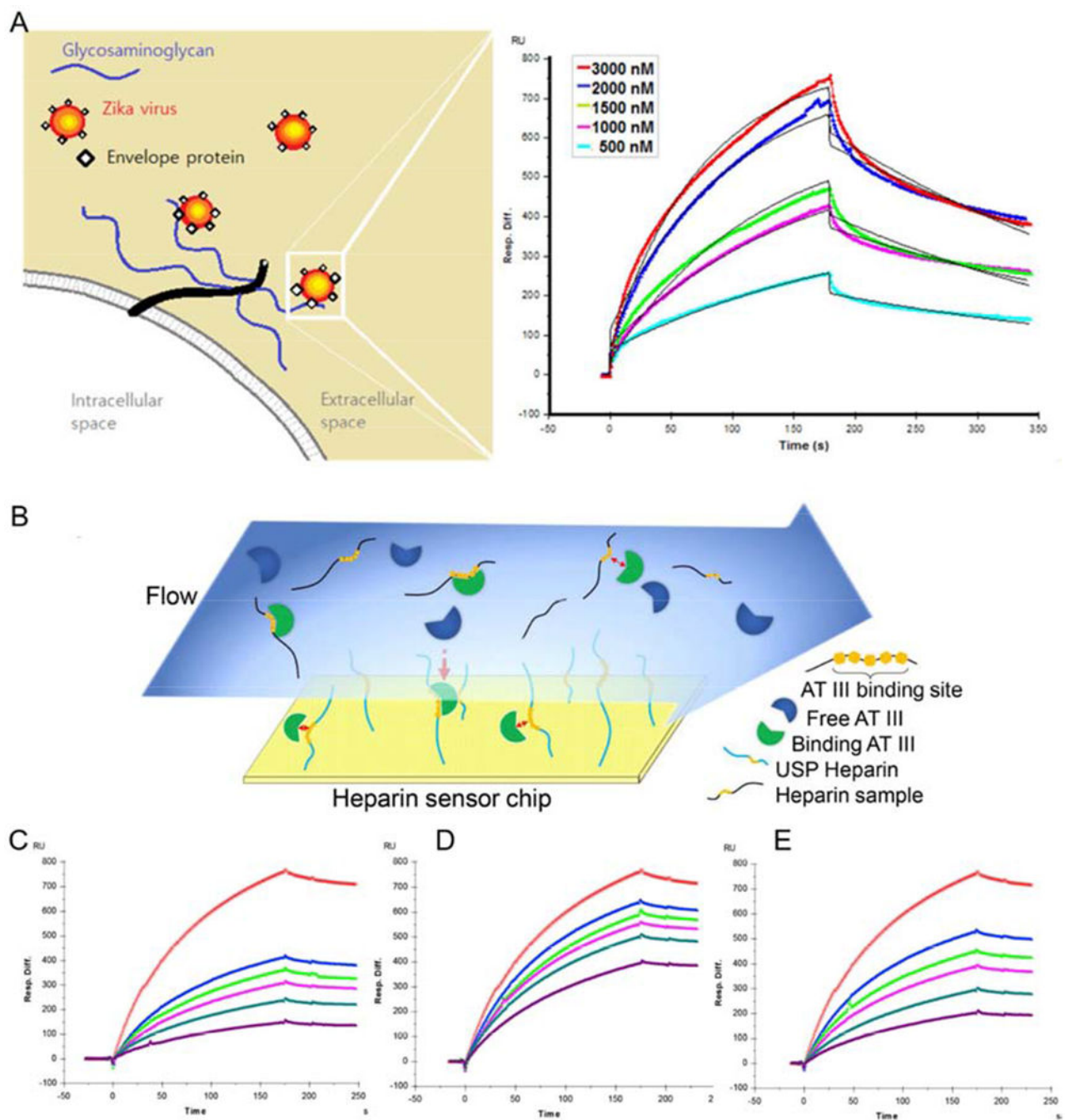


Figure 9. (A) Sensorgrams of interactions between ZIKV E protein and UFH. Copyright 2017 American Chemical Society. (B) Diagram of SPR solution competition experiment for anticoagulant activity measurement of heparin samples; (C) to (E): SPR sensorgrams of AT binding to heparin surface competing with different heparin samples, where (C): USP UFH standard; (D): LMWH (E): bovine lung derived UFH. Copyright 2017 Elsevier.

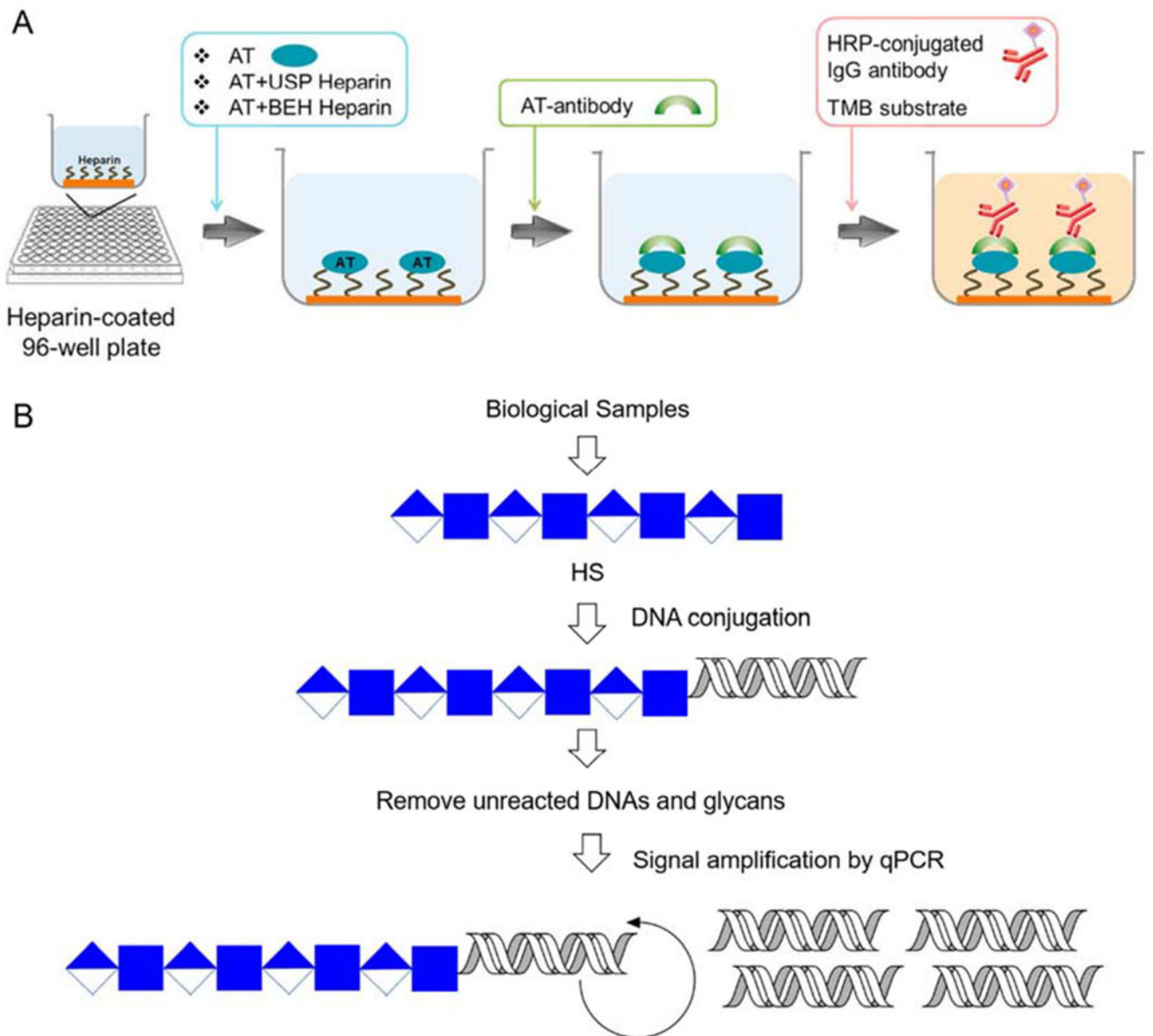


Figure 10.

(A) Schematic representation of high-throughput method for in-process monitoring 3-O-sulfation in bioengineered heparin synthesis. Copyright 2019 Elsevier. (B) Schematic representation of glyco-qPCR. Target carbohydrates (GAGs) from biological samples can be conjugated with different DNA “bar code”, followed by removing unreacted DNA and detecting the corresponding GAG-DNA conjugates with amplified signals (qPCR).

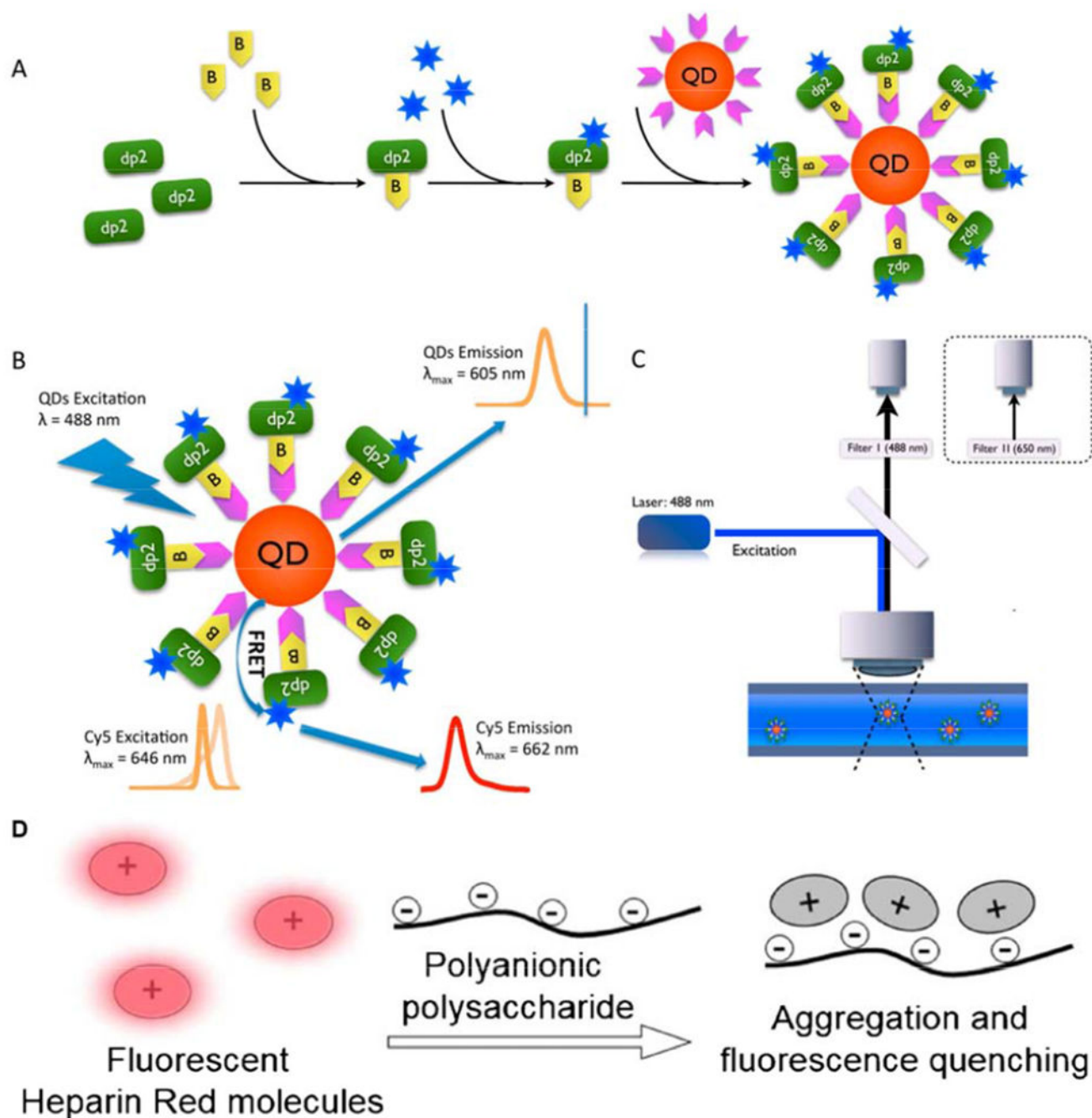


Figure 11.

Scheme of the FRET system construction in this study. (A) Conjugation scheme of the FRET system. (B) The FRET donor, QD, is excited with a laser at 488 nm; because Cy5 dye, located on the same disaccharide, is close, FRET occurs and Cy5 is excited by emission from the QD, and the emission of Cy5 is then detected. (C) CE-LIF instrumental setup for FRET detection. A 488 nm (filter I, donor-acceptor channel) and 650 nm (filter II, acceptor channel) band-pass filter was used for FRET detection. Copyright 2013 American Chemical

Society. (D) Schematic representation of fluorescence quenching of the molecular probe Heparin Red in the presence of polyanionic polysaccharides. Copyright 2016 Springer.

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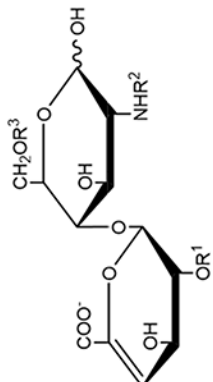
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Structures of heparin/HS disaccharide standards are shown where UA corresponds to deoxy- α -L-threo-hex-4-enopyranosyluronic acid.

Table 1.



HS Disaccharides	Structure	R ¹	R ²	R ³	Molecular Weight
0S	UA(1,4)GlcNAc	H	Ac	H	379,1115
2S	UA2S(1,4)GlcNAc	SO ₃ ⁻	Ac	H	459,0683
6S	UA(1,4)GlcNAc6S	H	Ac	SO ₃ ⁻	459,0683
NS	UA(1,4)GlcNS	H	SO ₃ ⁻	H	417,0577
2S6S	UA2S(1,4)GlcNAc6S	SO ₃ ⁻	Ac	SO ₃ ⁻	539,0251
NS2S	UA2S(1,4)GlcNS	SO ₃ ⁻	SO ₃ ⁻	H	497,0145
NS6S	UA(1,4)GlcNS6S	H	SO ₃ ⁻	SO ₃ ⁻	497,0145
TriS	UA2S(1,4)GlcNS6S	SO ₃ ⁻	SO ₃ ⁻	SO ₃ ⁻	576,9713