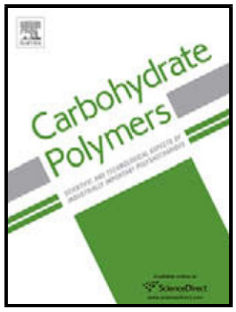

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Recent advances in the analysis of polysialic acid from complex biological systems

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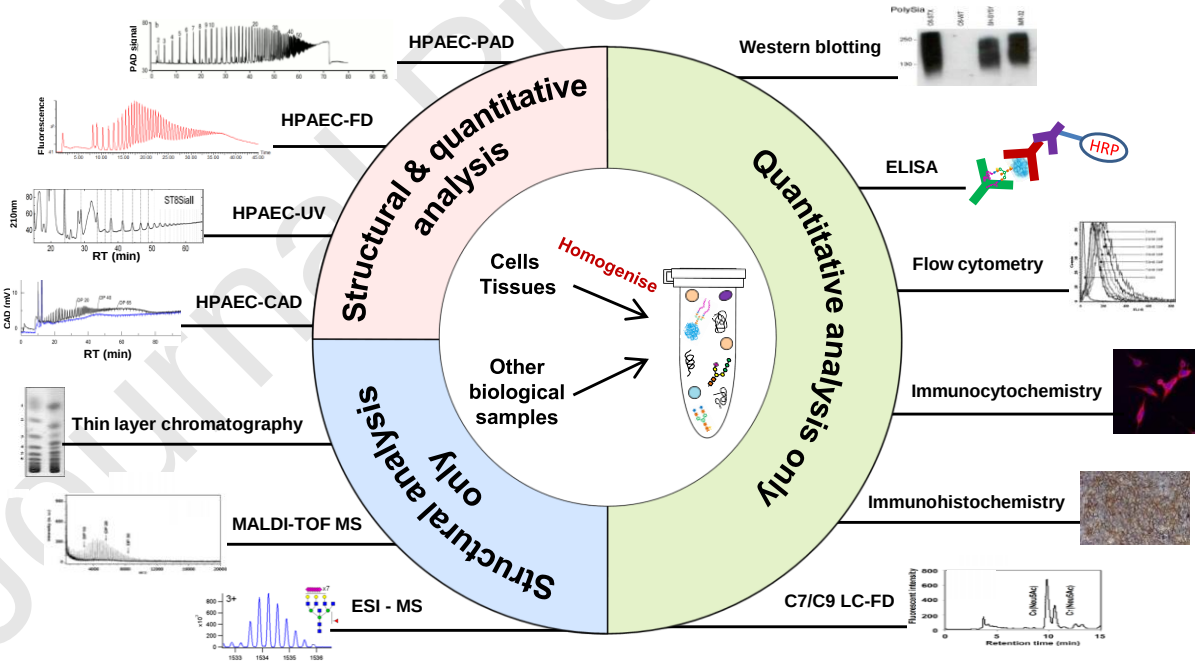
Graphical Abstract



Highlights

- Polysialic acid is of significant interest due its roles in biology and disease
- First systematic review of approaches for extraction and characterisation of polysialic acids from biological matrices
- Approaches to analysis of the degree of polymerisation of polysialic acid evaluated
- Quantitation considered, including accuracy and limit of detection

Abstract

Polysialic acid (polySia) is a unique, well-characterised carbohydrate polymer highly-expressed on the cell surface of neurons in the early stages of mammalian brain development. Post-embryogenesis, it is also re-expressed in a number of tumours of neuroendocrine origin. It plays important roles in modulating cell-cell, and cell-matrix adhesion and migration, tumour invasion and metastasis. Techniques for structural and quantitative characterisation of polySia from tumours and cancer cells are thus essential in exploring the relationship between polySia expression levels and structural and functional changes associated with cancer progression and metastasis. A variety of techniques have been developed to structurally and quantitatively analyse polySia in clinical tissues and other biological samples. In this review, analytical approaches used for the determination of polySia in biological matrices in the past 20 years are discussed, with a particular focus on chemical approaches, and quantitative analysis.

Keywords: Polysialic acid, glycan analysis, glycan quantitation**.**

Abbreviations:

PolySia = polysialic acid

Sia = sialic acid

DP = degree of polymerisation (of polysialic acid)

NCAM = neural cell adhesion molecule

HPLC = high performance liquid chromatography

MS = mass spectrometry

FD = fluorometric detection

ELISA = enzyme link immunosorbent assay

WB = western blotting

1. Introduction

Polysialic acids (polySia) are linear carbohydrate polymers, comprised of negatively-charged 5-*N*-acetylneuraminic acid (Sia, Neu5Ac) residues (**Fig. 1A**), linked uniquely through α-2,8- (predominantly), α-2,9- glycosidic bonds or α-2,8 / α-2,9 alternating- glycosidic bonds (**Fig. 1E-1G**) (Chao, Chuang, Chiou, & Liu, 1999; Janas & Janas, 2011). Besides the predominant Neu5Ac-containing polySia, other Sia units are found in polySia polymers in nature, namely: 5-*N*-glycolylneuraminic acid (Neu5Gc) (**Fig. 1B**) and deaminated-neuraminic acid (KDN) (**Fig. 1C**) (Sato et al., 1993). The chain length (or the degree of polymerisation, DP) of polySia varies from 8 to 400 monomer units (Nakata & Troy, 2005). PolySia is an important post-translational modification to many cell adhesion molecules, (Nakata & Troy, 2005; Muhlenhoff, Rollenhagen, Werneburg, Gerardy-Schahn, & Hildebrandt, 2013), the most abundant and well-studied of which is the neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily (Muhlenhoff et al., 2013). PolySia is attached via the fifth and sixth N-glycans present in the Ig5 domain of NCAM, its associated negative charge increasing the hydrodynamic radius of the

extracellular part of NCAM, thereby attenuating the adhesive forces between NCAM and other cell adhesion molecules (Colley, Kitajima, & Sato, 2014). By regulating the interactions of proteins on the surface of neighbouring cells, polySia plays a key role in many biological processes, including cell migration, synapse formation and functional plasticity within the nervous system (Colley et al., 2014; Schnaar, Gerardy-Schahn, & Hildebrandt, 2014). In mammals, polySia is highly-abundant in the developing stages of the embryonic nervous system, but its expression dramatically decreases in maturity (Finne, 1982; Bruses & Rutishauser, 2001). In adulthood, polySia expression is restricted to discrete areas of the brain associated with the regulation of synaptic plasticity (Finne, 1982; Angata et al., 1997). Besides its roles in neuronal development, polySia also exerts important roles in cell growth through binding interactions with cell and growth factors, such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) (Vutskits et al., 2001; Kanato, Kitajima, & Sato, 2008; Ono, Hane, Kitajima, & Sato, 2012). These, and roles in the development many other organs are comprehensively reviewed in (Colley et al., 2014; Galuska, Lutteke, & Galuska, 2017). In addition, recent studies reveal that polySia found in plasma is implicated in modulation of the immune system, *via* binding to histones and lactoferrins released during the neutrophil extracellular traps (NETs) (Ulm et al., 2013; Galuska, Galuska, et al., 2017; Zlatina, 2017; Zlatina et al., 2018; Kuhnle et al., 2019; Zlatina & Galuska, 2019).

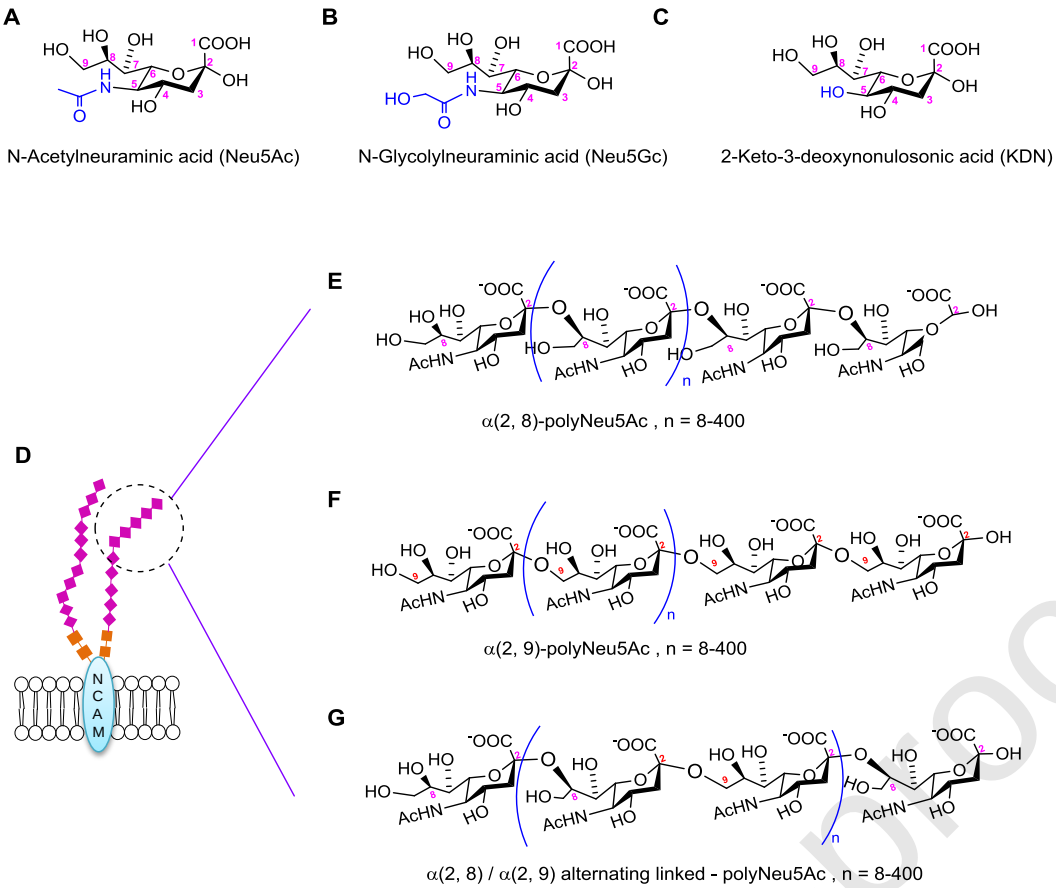


Fig. 1. Structure of sialic acid (A-C) and polysialic acid (E-G). Structural difference between major sialic acid family members are highlighted in blue. PolySia (D) can be linked by α(2,8)-linked (E), α(2,9)-linked (F) or α(2,8)/ α(2,9) alternating linked (G) - glycosidic bonds.

Furthermore, in recent years, numerous oncological studies have also revealed that polySia is expressed at high levels in a number of cancers principally of neuroendocrine origin, where it is involved in cancer cell growth, migration, invasion and metastasis (Falconer, Errington, Shnyder, Smith, & Patterson, 2012). The high expression level of polySia in tumours is strongly associated with poor clinical prognosis although the full extent of the role of polySia in cancer development and progression has yet to be elucidated. It is therefore a critical task to further explore the bioactivity, structure, and expression levels of polySia in tumours. However, due to the polydispersity, negative charge, relative chemical fragility, diversity of abundance, and the complexity of biological matrices, accurate structural characterisation and quantitative analysis of polySia from biological samples remains highly challenging. This is despite many rapid and

sensitive chemical and immunological techniques for polySia analysis having been developed in recent decades (Galuska, 2013).

In this review, analytical techniques available for structural characterisation and quantification of native polySia from biological matrices have been evaluated, together with their advantages and/or disadvantages in meeting these goals. In addition, strategies for the extraction and purification of polySia from biological matrices have also been considered.

2. Sample preparation for polySia analysis

For precise structural determination of polySia by chromatography and mass spectrometry, a pre-requisite and critical step is the isolation and enrichment of polysialylated proteins from biological matrices and subsequent polySia release from the parent glycoproteins. The latter has posed, and continues to pose, a very significant challenge. The chemical fragility of internal α-2,8 / α-2,9 glycosidic bonds means that it is inevitable that some degree of degradation of polySia occurs prior to analysis, depending on the method of polymer release. While isolation and enrichment of polySia might not be necessary for immunoassays, these processes can improve detection sensitivity, particularly for studying low-abundance polySia and polysialylated-glycoproteins. In this section, methodologies for isolating and purifying polySia, polysialylated proteins and polySia liberated from glycoproteins are each considered.

2.1 Isolation & enrichment of polySia and polySia-glycoconjugates

This sub-section summarises well-established isolation and purification techniques used for polySia analysis, involving conventional organic precipitation approaches and immunoaffinity-based purification. These are illustrated in **Fig. 2**.

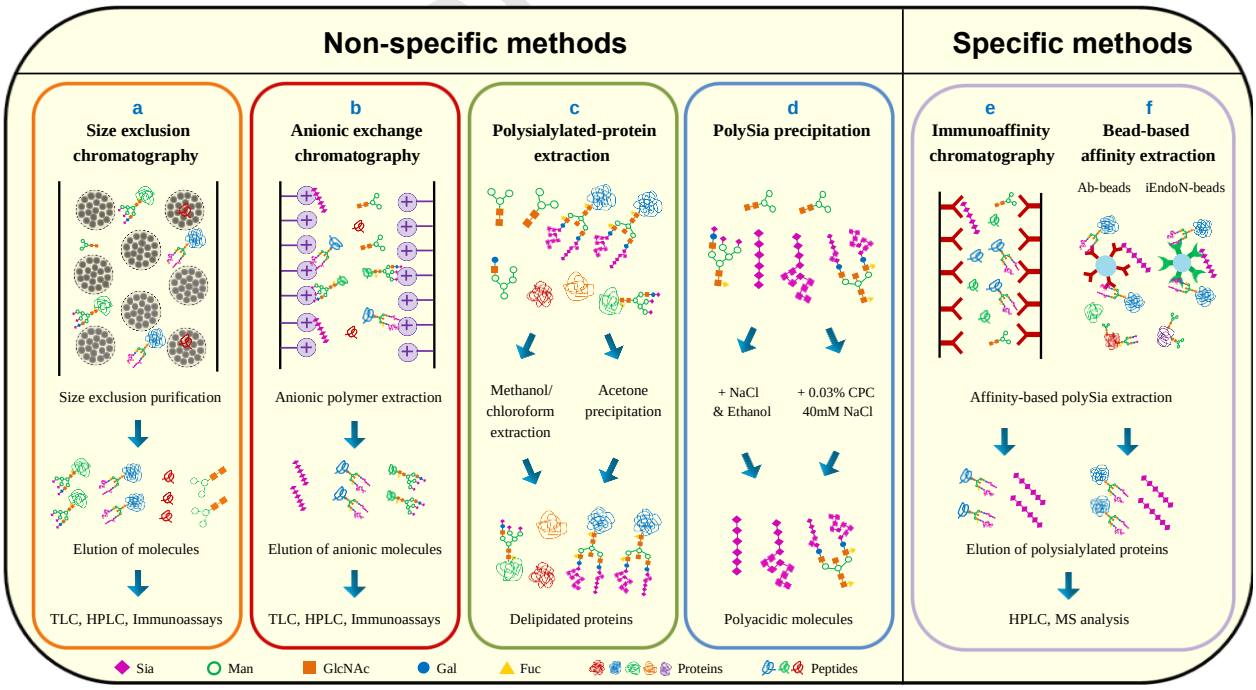


Fig. 2. Summary of methodologies for the extraction and purification of polySia and polySia bioconjugates from complex biological matrices.

2.1.1 Size exclusion chromatography

Size exclusion chromatography (SEC) is a conventional approach to fractionate polySia-bound glycoprotein from small molecules. It separates molecules according to their hydrodynamic volume and molecular size (Saraswat et al., 2013). This is achieved due to the presence of porous polymeric resins containing numerous microscopic holes in the chromatographic stationary phase of SEC, allowing smaller molecules to pass through. Larger molecules therefore elute faster than smaller molecules (Berek, 2010). By applying this approach, polysialylated proteins can be separated from other biological matrices such as lipids and free polySia polymers (**Fig. 2a**). Besides SEC, polysialylated proteins can also be separated from small molecules in biological matrices such as cell lysates by ultrafiltration cut-off membranes with a pore-size of 10 kDa (Guan, Wang, & He, 2015). In this way, molecules smaller than 10 kDa are removed. Alternatively, ultrafiltration-based membranes can also be used to de-salt and concentrate free polySia from biological matrices (Rode et al., 2008; Liu, Zhan, Wu, Lin, & Yu, 2010; Ehrit et al., 2017). As examples, Rode et al. and Liu et al. both employed 10 kDa ultrafiltration membranes to concentrate biosynthesised polySia from large amounts of *E.coli* broth culture before further purification of polySia (Rode et al., 2008; Liu et al., 2010). Compared to SEC, ultrafiltration membranes are a fast and straightforward technique, widely-used for desalting and concentrating samples. Limitations of ultrafiltration membranes include the risk of losing hydrophobic proteins which might bind to the membranes, and that it is not possible to separate carbohydrates and proteins of similar molecular weight.

2.1.2 Anion exchange chromatography

Anion exchange chromatography (AEC) is also a classical approach to purifying free polySia and related bioconjugates, such as polysialylated-proteins, peptides and glycans from neutral and alkaline components in complex matrices. It utilises the principle of electrostatic interactions between charged molecules. The stationary phase of an anion exchange chromatography column is covalently-coated with positively-charged materials to capture negatively-charged molecules in the mobile phase (Khan, 2012). The captured negatively-charged molecules can then be eluted by buffer of high ionic strength, such as Tris-HCl buffer (pH 7.6) containing 1 M sodium chloride. As relatively weak acids, free polySia and polysialylated bioconjugates can be isolated by weak anion exchange chromatography from neutral and basic molecules in complex biological environments (**Fig. 2b**) (Shimamura, Endo, Inoue, & Inoue, 1983; Sato et al., 1993; Sato et al., 1995; Zhang, Inoue, Inoue, & Lee, 1997; Lin, Inoue, & Inoue, 1999; Miyata, Sato, Kitamura, Toriyama, & Kitajima, 2004; Miyata et al., 2006). A weak anion exchange DEAE-Toyopearl 650 M column (2.2 x 15 cm) was used to purify fractionated peptides containing α-2,9-linked polySia from sea urchin sperm, with up to 20 Sia units (Miyata et al., 2004; Miyata et al., 2006). AEC can also be used to fractionate free polySia polymers into different chain lengths based on the number of negative charges present. For example, a weak anion exchange column DEAE-Sephadex A-25 chromatography (1 x 13 cm) can be used to fractionate α-2,8-linked oligo- and polySia released from glycoproteins from fish eggs (Sato et al., 1993). Purified disialic acid and polySia-containing 11 Sia units from polySia was isolated from *N. meningitides C* by applying strong ion exchange chromatography (Ming, Caro, Lanz, Vionnet, & Vann, 2019).

The main limitation of anion exchange chromatography in this context is that it cannot be used to distinguish polySia and polysialylated bioconjugates from each other, and from other acidic molecules, such as poly-nucleic acids and gangliosides. Therefore, to achieve improvements in separation of these molecules, AEC is often applied, in combination with other separation techniques such as size exclusion chromatography. In addition, since the presence of high amounts of salts in the samples might affect downstream analyses, it is usually applied in

combination with other purification strategies, such as size exclusion chromatography or reverse-phase chromatography, to enrich free polySia or polysialylated bioconjugates.

2.1.3 Organic solvent delipidation of polysialylated proteins

Organic solvent extraction is the most straightforward approach for isolating proteins. Polysialylated-proteins can be separated from membrane lipid content including gangliosides by chloroform/methanol/aqueous buffer extraction (a ratio of 4:8:3, v/v/v, is commonly used) (**Fig. 2c**) (Kudo et al., 1996; Inoue & Inoue, 2001a; Poongodi et al., 2002). Chloroform/methanol/water extraction is an efficient technique for extracting gangliosides and lipids from biological samples (Svennerholm & Fredman, 1980). It was first applied in delipidating polysialylated-glycopeptides from embryonic chick brain homogenates by the Inoue group (Kudo et al., 1996). After centrifugation, lipids are dissolved in organic solvent, while proteins and peptides are precipitated as a pellet. As an alternative to chloroform/methanol/water extraction, acetone has also been used to precipitate polysialylated-proteins from biological samples at low temperatures (4 °C) (Simon et al., 2013). The specific challenge with organic solvent precipitation is the difficulty in solubilising the precipitated polysialylated proteins. To solve this problem, Inoue used buffered non-ionic detergents, including 0.5 % Igepal CA-630 or Triton X-100, to solubilise the delipidated proteins from homogenised cell lysates and tissues, with subsequent sonication and incubation (0.5-1 h at 37 °C), which further facilitated protein solubilisation (Inoue & Inoue, 2001b, 2001a).

2.1.4 Organic solvent precipitation of free polySia

Besides use in precipitating polysialylated-proteins, organic solvents such as ethanol have also been used to precipitate free polySia molecules from biological matrices (Rode et al., 2008; Sugumaran et al., 2012; de Vries et al., 2018). Similar to other anionic nucleotide polymers, binding with positive ions significantly reduces the solubility of polySia in ethanol, resulting in polySia precipitation. The more Sia residues the polymer contains, the more rapidly the polymer

can be precipitated. Ethanol precipitation is often used in the purification of polySia from fermentation broths. For instance, the Schepera group employed 80% ethanol to separate large quantities of polySia in the presence of sodium chloride, with 95% recovery, after pre-treatment by acetone precipitation and quaternary ammonium precipitation (Rode et al., 2008; Sugumaran et al., 2012). Key advantages of organic solvent precipitation of free polySia are the ease of use and low cost. However, it is worth noting that ethanol may not be efficient in precipitating short-chain polySia. Moreover, as a non-specific approach, ethanol precipitation cannot separate polySia from other polyanionic polymers, such as DNA and gangliosides.

2.1.5 Quaternary ammonium salts precipitation of polySia

As a polyanionic molecule, polySia can also be precipitated by cationic quarternary ammonium salts such as cetylpyridinium chloride (CPC) and hexadecyltrimethylammonium bromide (cetrimonium bromide, CTAB) to form aqueous insoluble complexes, in low ionic strength solution at a pH < 9, while proteins and neutral carbohydrates remain in solution (Kitazume, Kitajima, Inoue, & Inoue, 1992; Sugumaran et al., 2012). The polySia precipitates are re-dissolved in high-salt solutions. In a manner similar to that described for ethanol precipitation, polySia with larger numbers of negative charges can be easily precipitated. Therefore, by adjusting the concentration of CPC or CTAB as well as the solution ionic strength, polySia can be fractionated. For instance, Kitazume et al. successfully isolated polySia with more than 7 Sia residues, and more than 12 Sia units were selectively precipitated by CPC (0.033%) in the presence of NaCl, (40 mM and 100 mM respectively), while the precipitated polySia was recovered by increasing the salt concentration (to 200 mM) (Kitazume et al., 1992). This approach is used often in combination with organic precipitation to purify large quantities of polySia from fermentation media. For instance, large scale purification of polySia was achieved by separation from *E.coli* fermentation broth by performing a sequential precipitation with acetone-CTAB-ethanol (Rode et al., 2008; Liu et al., 2010). The advantage of this approach is the low cost. The main disadvantages are the time-consuming nature of the process, and the challenge of efficient

separation of polySia from other polyanions such as nucleic acids. Moreover, the approach requires further de-salting processes to remove the high amount of salts introduced during the precipitation. Despite these difficulties, this approach is adequate to serve other downstream analytical techniques.

2.1.6 Immunoaffinity-based purification of polySia bioconjugates

Immunoaffinity-based techniques are highly-specific and efficient tools to separate target biomolecules from complex systems. The technique relies on the capability of a solid support surface incorporating antibodies or other biological probes that recognise and capture specific biological targets. It includes a "capture-wash-elute" process to purify target analytes. By employing different types of solid support, the techniques can be divided into chromatography-based techniques and bead-based techniques, each of which will be explored below.

2.1.6.1 Biological probes for free polySia and polysialylated bioconjugates

Two major types of biological probe have been used to specifically identify free polySia and polySia-bound bioconjugates, namely anti-polySia antibodies, and inactived endoneuraminidase-N (EndoN). Although various anti-polySia antibodies are available nowadays, the main antibody used for immunoaffinity capture of polySia and its carriers is the anti-polySia monoclonal mAb 735, due to its high specificity. The monoclonal mAb 735 can only recognise polySia containing more than 11 Sia units (Sato & Kitajima, 2008). Since its binding to polySia is based on electrostatic interaction, it exhibits high affinity towards long-chain polySia (DP > 100) and the binding affinity can be inhibited in the presence of high concentrations of salts, e.g. more than 150 mM NaCl (Hayrinen et al., 2002). On the other hand, endoneuraminidase-N (EndoN) is an enzyme found in the *E. coli* K1 bacteriophage that can specifically hydrolyse polySia: it recognises and binds to specific regions of polySia of 5-10 Sia units, cleaving the internal (*endo*) α-2,8 glycosidic bonds. In the case of enzymatically inactive EndoN, the mutant enzyme binds to polySia with strong affinity, but has no hydrolytic activity (Jakobsson et al., 2007). Inactive

EndoN has higher affinity towards polySia with a K_d of 1.9 nM as compared to 7 nM for mAb 735 (Jakobsson, Schwarzer, Jokilammi, & Finne, 2015). Inactive EndoN has thus become a popular alternative.

2.1.6.2 Immunoaffinity chromatography

In immunoaffinity chromatography (IAC), the biological probes are coated on the surface of a chromatographic matrix (i.e. on the stationary phase) to recognise the target in a mobile phase. Coating with specific anti-polySia antibodies, IAC has been successfully used to purify polySia-containing peptides and polysialylated glycans from a wide range of cell lysates and clinical tissues (**Fig. 2e**) (Hayrinen et al., 2002; von Der Ohe et al., 2002; Wuhrer et al., 2003; Galuska, Geyer, Gerardy-Schahn, Muhlenhoff, & Geyer, 2008). As an example, polySia-containing peptides have been successfully eluted from antibodies by strong alkaline solutions such as triethylamine (100 mM) (Galuska et al., 2008). The merit of this technique is high specificity, resolution and large capacity, in contrast to more non-specific chemical purification tools, but it nevertheless carries the earlier discussed risk of some degree of chemical degradation, due to the alkaline conditions. Due to the large sample capacity, IAC is ideal for purifying polySia and polysialylated-bioconjugates from large sample volumes, such as tissue homogenates, for downstream analysis which requires high standard of sample purity, effectively enriching the sample. For instance, the Galuska group successfully purified polySia-NCAM proteins and polySia glycopeptides from mouse brain by applying a polySia-specific monoclonal antibody mAb 735-coated protein A-sepharose immunoaffinity chromatography process to study the glycosylation patterns of the polysialylated-proteins and relevant polySia structures. However, limitations of the technique are that the cost is significantly greater than that for chemical tools, and reliance upon the quality of the antibody in question (mAb 735 is widely used).

2.1.6.3 Immunoprecipitation

Immunoprecipitation is a conventional technique to extract proteins of interest from complex matrices. In this technique, the anti-polySia antibody is normally applied first to the biological sample to capture the polySia conjugates. Subsequently, after incubation with protein A/G-coated agarose beads, the bead-antibody-polySia conjugate complexes are separated from impurities by centrifugation. The polySia conjugates are then eluted by an alkaline buffer. Immunoprecipitation offers high specificity, a large binding capacity and low cost. It is often used for purifying polySia conjugates in combination with Western blotting to investigate the molecular weight of polySia-proteins and of the carrier proteins. For instance, anti-polySia antibody-protein G-Sepharose beads were used for capturing polysialylated proteins from mouse bone marrow lysates followed by identification of their carrier protein by Western blotting (Drake et al., 2008). However, due to the large diameter (50 - 150 μm) and the porous nature of agarose beads, the principal limitation of immunoprecipitation is the difficulty in accessing antibody-complexes trapped in the agarose pores negatively affecting yield, in addition to the risk of degradation by the alkaline buffer.

2.1.6.4 Dynabead-based affinity purification

Magnetic beads have received increasing attention for purification of biological analytes. Here, the beads comprise an inner ferro-magnetic core and a surface coated with polymer material, which can be functionalised with biological probes that allow specific binding to analytes of interest (Karimi, Karimi, & Shokrollahi, 2013). Dynabeads are described as *superparamagnetic spherical polymer particles with a uniform size and a consistent, defined surface for coupling with biological probes.* With regards purifying polySia and related bioconjugates, the surface of the Dynabeads is covalently-bound with either an anti-polySia antibody or inactivated EndoN. (**Fig. 2f**). Following capture, the bead-antibody-polySia complex is isolated and conveniently attracted to the tube wall by applying a ferro-magnet, allowing other components to be discarded. After washing, the purified polySia or polySia conjugates can be released from the bead-probes under alkaline conditions. The primary benefit of Dynabead-based affinity purification is the high

specificity for polySia. Moreover, the Dynabead-based approach also has the advantages of ease-of-use, low level of contaminants, fast and amenable to high throughput. However, as the technique is normally restricted to low volume sample tubes, the approach is limited to small-scale sample purification, e.g. of complex matrices, such as cell lysates.

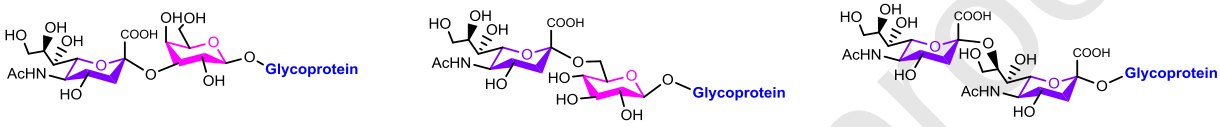
2.2 Release of polySia from glycoproteins

Cleavage of polySia from glycoproteins is a prerequisite for characterisation of the sugar polymer by chemical analytical approaches. Strategies to release intact polySia chains from carrier proteins can be classified into chemical and enzymatic approaches. The chemical approach is further sub-divided into 'mild' hydrolysis and alkaline-based release methods; which are generally applicable and inexpensive. As discussed earlier, these techniques carry significant risk of polymer degradation, due to the sensitivity of the internal α-2,8 / α-2,9 bonds to chemical hydrolysis outside of neutral pH conditions. The degree to which this occurs is dependent on the specific conditions employed, but a methodology for truly accurate determination of polySia chain length remains elusive. The enzymatic release approach is more specific but more limited in its application.

2.2.1 Mild acid hydrolytic release of polySia

PolySia is cleaved from glycoconjugates in a mildly acidic environment, typically provided by trifluoroacetic acid (20 mM, pH 2.0). The mechanism of release of intact polySia chains from glycoconjugates is based on the acid lability of the α-2,6 or α-2,3 Sia-galactose (Gal) glycosidic bonds between polySia and its bioconjugate (**Fig. 3**). which are more sensitive to mild acid attack than the polySia (Sia-Sia) α-2,8- glycosidic bonds within the polySia polymer structure (Inoue & Inoue, 2001b; Nakata & Troy, 2005). Moreover, the lactonisation of polySia in acidic conditions also contributes to the stability of polySia chains in mildly acidic conditions (Zhang & Lee, 1999). However, partial hydrolysis of internal Sia-Sia α-2,8 bonds, which are more sensitive to acid hydrolysis, can still occur in long-chain polySia in either the cleavage from the glycoprotein or labelling steps (Inoue & Inoue, 2001a; Nakata & Troy, 2005), providing a major challenge to this

approach. While not solving this problem completely, an attempt to minimise unwanted internal Sia-Sia α-2,8 bond cleavage during the subsequent fluorescent labelling step (discussed in section 3.2.3) was achieved using lower temperatures (4 °C), albeit requiring longer incubation times (Galuska, 2013). After mild acid hydrolysis, reactions are terminated by alkaline neutralisation to reverse the lactonisation within polySia (Galuska, 2013). This approach has proved to be efficient. However, despite optimisation of temperature and reaction time, the problem of polySia of very long DP being partially hydrolysed during the conditions of release from the glycoprotein remains. That said, this mild acid hydrolysis methodology has been commonly employed, with the advantages of ease-of-use, universal application and low cost.



α-2,3-Sia-Gal linkage α-2,6-Sia-Gal linkage α-2,8-Sia-Sia linkage

Fig. 3. Structure of Sia linkage in glycoproteins. Sia sugar unit is presented in purple and Galactose sugar unit is in pink.

2.2.2 Alkali / borohydride release of polySia

The use of sodium borohydride represents another non-enzymatic approach to the release of polySia from glycoproteins (Angata et al., 1994; Miyata et al., 2004; Miyata et al., 2006). Under mild alkaline conditions, polysialylated O-GalNAc-linked glycans can be released from glycoproteins by β-elimination (Fukuda, 2001). The released polysialylated O-linked glycan is then reduced by borohydride to a base-stable polysialoglycan alditol, preventing an unwanted elimination reaction and polymer isomerisation (Fukuda, 2001) (**Fig. 4**). After cleavage, the polysialoglycan alditols with different DPs can be separated by high performance anion exchange chromatography (HPAEC) and visualised by UV absorbance. This approach is of value for analysis of O-linked polysialylated-glycans; N-linked polysialylglycans are somewhat refractory to hydrolysis under mild alkaline. Alkali/borohyride release of polySia was successfully used by

Miyata et al. (2004) to study the structure of O-linked polysialoglycans from sea urchin sperm. Since this approach does not lead to the partial internal self-cleavage of α-2,8-linked Sia-Sia bonds in polySia, it enables the study of polySia DP with accuracy. However, a disadvantage is the high salt content remaining following the alkali/borohydride reaction, which can affect some downstream investigations, such as polymer separation by anion exchange chromatography. A desalting process is therefore likely to be necessary prior to chemical analysis (Makino et al., 2000). Another limitation is that the formation of the alditol makes the polymer difficult to be derivatised, and thus limits the choice of strategies requiring further derivatisation prior to analysis.

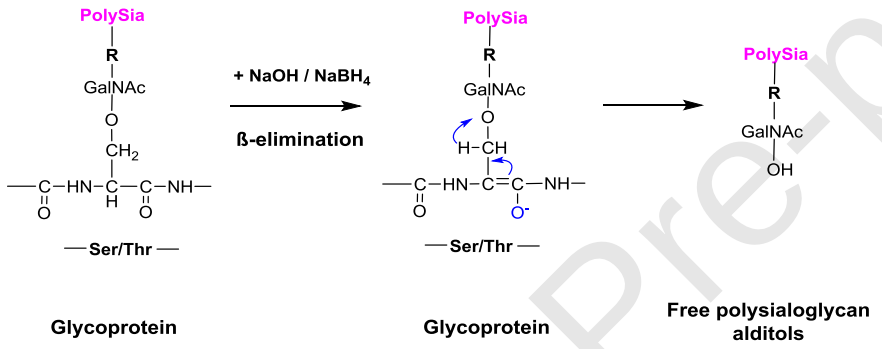


Fig. 4. β-elimination reactions of O-linked polysialylated-glycans.

2.2.3 Enzymatic release of polySia

To maximally avoid Sia-Sia partial hydrolysis of polySia chains during mild acid hydrolysis, wholly enzymatic approaches are required. An approach to liberate polySia from glycoconjugates has been established using endo-β-galactosidase, which cleaves the N-acetyllactosamine (galactose (1→4) N-acetylglucosamine) repeating linkages in the core N-glycan structure between polySia and its glycan carriers (Nakata & Troy, 2005). The enzymatic reaction is performed in mildly acidic conditions (pH 5.9) at 37 °C for 12 hours (Nakata & Troy, 2005). The major advantage of endo-β-galactosidase cleavage is the highly specific release of intact polySia chains

from glycoproteins, apparently without causing detectable loss or degradation of the long-chain polySia (Nakata & Troy, 2005; Galuska, 2013). This is highly useful for sensitive and accurate structural characterisation of long-chain polySia. In addition, endo-β-galactosidase-mediated polySia cleavage can be used for quantitative analysis of low-abundance polySia from complex matrices. PolySia DP 400 and 140 Sia residues has been successfully detected from mouse neuroblastoma cells and human NK cells, respectively (Nakata & Troy, 2005; Drake et al., 2008). However, a significant limitation to the technique is that not all polysialylated N-glycans contain the poly-N-acetyllactosamine structure for endo-β-galactosidase to recognise and cleave (Nakata & Troy, 2005; Galuska et al., 2006). Moreover, it is important to consider the impact of pH, temperature and reaction time on polySia thermal stability, since polySia has been reported to be unstable in a pH 5.6 environment at 37 °C for 24 hr (compare this to that reported by Nakata et al. above), whereas the polymer was stable at neutral pH at 37 °C for more than 1 day (Manzi, Higa, Diaz, & Varki, 1994; Inoue, Lin, Lee, & Inoue, 2001). Therefore, small variations in these parameters, especially in pH of the buffer, can lead to significant changes in outcome from the enzymatic reaction, and the risks of chemical damage to very long chain polySia remain.

3. Structural characterisation of polySia from biological samples

Structural characterisation of polySia is an important part of the landscape of polySia analysis. In this section, analytical tools employed in characterising the degree of polymerisation of polySia are reviewed, with a focus on chemical-based techniques. Table 1 summarises the application of chemical analytical techniques to the study of polySia from biological samples.

3.1 Thin-layer chromatography-based analysis

Thin-layer chromatography (TLC) was one of the first techniques reported to analyse and/or separate polySia from biological matrices (Finne & Makela, 1985; Kitazume et al., 1994; Sato et

al., 1995; Miyata et al., 2004), exploiting the partitioning properties of an analyte between a polar stationary phase (silica) and a non-polar organic solvent mobile phase. TLC analysis reveals that polySia with high DP, which is polar, migrates more slowly than oligoSia. Resorcinol can be used to visualise the separated sugars. Since TLC separation is based on the polarity of analytes, this technique can be also applied to study the degree of polymerisation of oligoSia composed of different Sia isomers. For instance, poly-Neu5Gc has been successfully separated from oligo-KDN from rainbow trout by TLC, (Sato et al., 1995) as the side chain at the carbon 5 position of Neu5Gc Sia is less polar than that in KDN (**Fig. 1 B, C**). Although TLC is a facile and inexpensive process compared to other separative analytical techniques, it has limitations, namely poor resolution of polySia with greater than 10 Sia units, and low detection sensitivity requiring at least 1 μg of analyte. The development of high performance chromatography-based fluorometric techniques (Kitazume et al., 1994; Galuska, 2013) has largely supplanted the use of TLC.

3.2 Chemical analysis based on high performance anion exchange chromatography (HPAEC)

High performance anion exchange chromatography (HPAEC) has been widely employed in the structural analysis of polySia, due to its high resolution in separating polySia of different chain lengths. As a weak acid, polySia binds to the stationary phase of an HPAEC column, and its binding capacity is proportional to its total number of negative charges. Thus, polySia can be effectively separated into various chain lengths by an HPAEC column in a single analysis. HPAEC can be coupled with a detector based on amperometry, fluorescence, UV absorbance or mass spectrometry to characterise and quantitate the polySia content.

3.2.1 Anion exchange chromatography-based pulsed amperometric detection (HPAEC-PAD)

Pulsed Amperometric detection (PAD) measures the change in electrical currents produced by electroactive analytes, which undergo a redox reaction on the surface of an electrode under a

repetitive triple-pulsed potential (Corradini, Cavazza, & Bignardi, 2012). PAD is an attractive analytical technique in the determination of polySia with higher degree of polymerisation, in combination with high performance anion exchange chromatography (HPAEC) at alkaline pH (Inoue, Lin, & Inoue, 2000; Richard, Buon, Drouillard, Fort, & Priem, 2016). PolySia with DP up to 90 could be quantitated from colominic acid (a commercially-available range of polySia chain lengths typically derived from *E. coli*) by using a PAD-2 detector coupled to a CarboPac PA-100 HPAEC column (Zhang et al., 1997). In addition, an ED-40 PAD coupled with a CarboPac PA-100 column was used successfully to detect polySia with a maximum DP of 50 in embryonic chick brains (Inoue et al., 2000). Regardless of these successes, the limitation of HPAEC-PAD is relatively poor sensitivity and poor selectivity requiring more than 10 μg of purified polySia samples to visualise polySia of DP more than 30 in a single analysis (Zhang et al., 1997).

3.2.2 Anion exchange chromatography with UV analysis (HPAEC-UV)

The degree of polymerisation of polySia can also be measured by UV absorbance at 210-214 nm, after separation by HPAEC, as has been elegantly demonstrated. (Chao et al., 1999; Miyata et al., 2004; Miyata et al., 2006; Kanato et al., 2008; Volkers et al., 2018). The UV absorbance of polySia at 210-214 nm is due to the amide bonds within polySia (i.e. the NHAc group in Sia monomer units). UV absorbance analysis can be applied to the determination of polySia released by alkali/borohydride treatment to study the polySia from O-linked glycoproteins (Miyata et al., 2004; Miyata et al., 2006). In addition, HPAEC-UV can also be used to study free polySia with a resolution of up to 25 Sia units, and to analyse the enzyme kinetics of polysialyltransferase (Kanato et al., 2008). The major benefit of UV absorbance for polySia DP analysis is that derivatisation of polySia prior to visualisation is not required, which is advantageous for studying long polySia chains. However, a serious problem with UV absorbance detection is poor selectivity using short UV wavelength (210 nm), in which other components in samples can also exhibit strong absorbance, resulting in high background noise during analysis. Therefore the technique demands high sample purity for assessment. Moreover, the choice of solvent can also have

significant impact on sensitivity. For example, buffer employing sodium chloride as the exchanger ion is the common choice used for HPAEC-UV analysis in all reported studies. This is probably due to its low absorbance in UV detectors, compared to buffers employing other ion exchanger salts, such as sodium nitrate and ammonium acetate. The need for at least 10 μg purified polySia for analysis by HPAEC-UV is a disadvantage, hence it is not frequently used.

3.2.3 Anion exchange chromatography with fluorometric analysis (HPAEC-FD)

Fluorometric analysis of polySia in combination with high performance anion exchange chromatography (HPAEC-FD) has been the most widely-employed method for studying the degree of polymerisation of polySia from biological samples. In this approach, polySia liberated from glycoconjugates is typically labelled with a fluorescent tag, frequently DMB (1,2-diamino-4,5-methylenedioxybenzene). DMB reacts with the reducing end of sialic acid residues under acidic conditions **(Fig. 5A)** (Galuska, 2013). Since this derivatisation process requires acidic conditions, the method is often applied in combination with mild acid hydrolysis to analyse polySia from glycoproteins, as a "one-pot" reaction (Lin et al., 1999; Inoue et al., 2000). In this process, once the polySia is cleaved from the glycoproteins, the free reducing ends of the sugar are exposed and react with DMB **(Fig. 5A)**. After DMB derivatisation, the reaction mixture is adjusted to alkaline to reverse and suppress lactone ring formation in the derivatised polySia, which readily occurs under acidic conditions. The multiple cationic charges associated with the derivatised polySia is critical for separation by HPAEC and the DMB derivitisation enables polySia to be visualised by fluorescence detection (Inoue & Inoue, 2001a) **(Fig. 5B)**. Since DMB derivatisation requires the carboxyl group and the free anomeric hydroxyl group (C-2 carbon of sialic acid), its use greatly improves selectivity for sialic acids over other carbohydrates. Furthermore, DMB-based fluorescence analysis offers high sensitivity from often very small quantities of polySia in limited available biological samples as compared to the amperometric analyses above. PolySia with DP > 90 has been visualised by DMB labelling/HAPEC-FD from as

little as 200 ng derivatised colominic acid has been reported (Inoue & Inoue, 2003). This approach has been widely-used for studying polySia from cells and tissues with detectable DP ranging from 18 to 60 (Inoue & Inoue, 2001a, 2001b; Poongodi et al., 2002; Yabe, Sato, Matsuda, & Kitajima, 2003; Simon et al., 2013; Nishimura et al., 2014; Kaese et al., 2015; Abe et al., 2017). However, the main challenge of mild acid hydrolysis coupled with HPAEC-FD is the potential partial hydrolysis of long-chain polySia during the hydrolysis reaction and DMB labelling, as noted by Inoue (Lin et al., 1999; Inoue et al., 2001). To minimise this, the DMB derivatisation reaction conditions have been extensively optimised by S. Inoue (Inoue et al., 2000; Nakata & Troy, 2005; Ulm et al., 2013). By performing the DMB labelling with biological samples at lower temperatures with longer periods of incubation, polySia of at least 60 Sia residues has been detectable from biological matrices (Inoue & Inoue, 2001a).

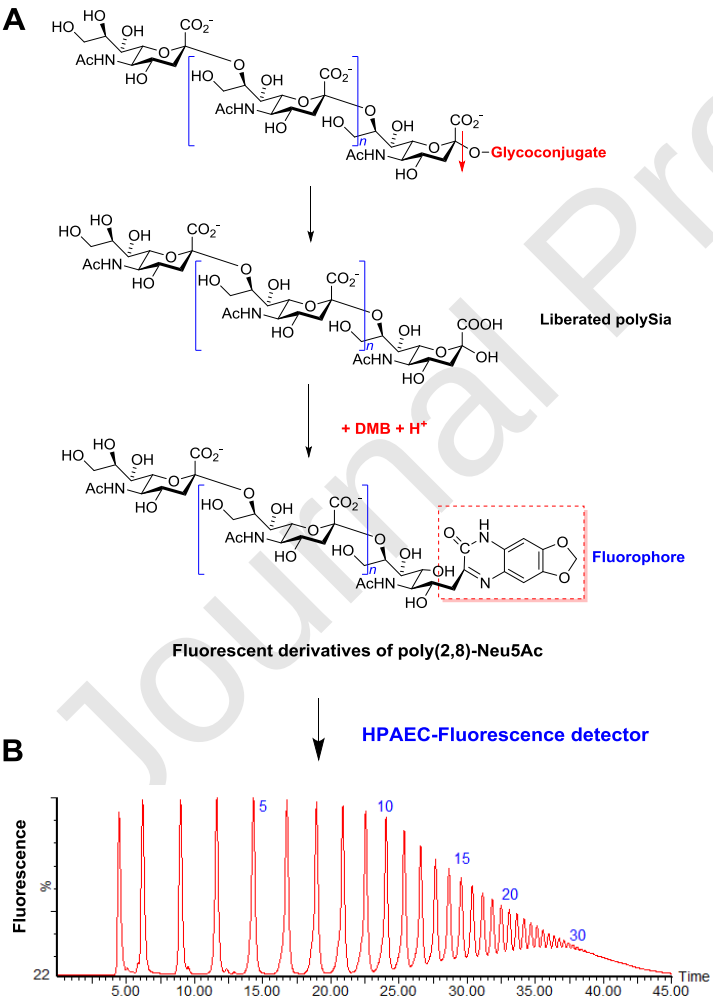


Fig. 5. PolySia derivatisation with HPAEC chromatography-fluorometric detection. (A) PolySia cleavage from glycoconjugates followed by DMB derivatisation in an acidic (pH ~2) environment. (B) Typical fluorescence chromatogram of DMB-derivatised polySia polymers separated based on chain length (DP; indicated in blue) on a HPAEC column.

3.2.4 Anion-exchange liquid chromatography with charged aerosol detection (CAD)

More recently, a novel detection method based on charged aerosol detection (CAD) was introduced as an alternative to the fluorometric approach to detect polySia polymers. CAD is a technique that has been widely applied in measuring non-volatile analytes in various fields (Vehovec & Obreza, 2010). The detection process initiates from the conversion of the LC eluents to aerosol droplets by employing a nebuliser in the presence of nitrogen. Subsequently, the resultant aerosols pass through an evaporation tube to exclude the solvent and volatile components, with the remaining non-volatile analytes turned into dry particles, in which the surface is then charged with positive ions after collision with positively-charged nitrogen. After removing the excess positive ions by passing through an ion trap, the number of positive charges in the particles is detected by an electrometer (Magnusson, Risley, & Koropchak, 2015). In this approach, the maximum DP of 25 µg of polySia polymer standards and purified polySia from *E.coli* detected by CAD was 65 and 130, respectively (Bossmann et al., 2019). Similar to UV detection of polySia, the main advantage of this technique is the lack of need to perform derivatisation of polySia prior to detection, so the partial hydrolysis of long-chain polySia will be avoided in this approach. The process is also less time consuming than other methods (Bossmann et al., 2019). However, compared to the fluorometric analysis approach, CAD detection of polySia is less specific and less sensitive. In addition, the choice of mobile phase for CAD detection is restricted to volatile buffers, and the presence of increased salt concentrations in buffer gradients can also cause result in decreased resolution of CAD, particularly for polySia of higher DPs (de Villiers, Gorecki, Lynen, Szucs, & Sandra, 2007; Bossmann et al., 2019). Therefore, CAD detection is suitable for

rapid qualitative analysis of polySia from large samples, but not for the sensitive determination of polySia chain length, or for small quantities. Moreover, due to the nature of CAD detection, this approach might require high standards of sample purity, compared to fluorometric detection.

3.2.5 Reverse-phase liquid chromatography with fluorometric analysis (RPLC-FD)

The degree of polymerisation of polySia obtained from biological sources can be indirectly measured by reverse-phase HPLC coupled with fluorometric analysis (RPLC-FD) of derivatised hydrolysates of polySia fractionated through HPAEC. In this approach, polySia is released from glycoconjugates by endo-β-galactosidase, and then subjected to HPAEC, to separate polySia polymers according to chain length (DP) (Nakata & Troy, 2005). The fractionated polySia is then hydrolysed to sialic acid monomers under strong acid conditions at high temperatures (e.g: 0.1 M TFA, 80 °C), followed by DMB fluorimetric derivatisation. By using RPLC-FD to measure the amount of derivatised Sia hydrolysates in each of the polySia fractions, the maximum DP and relative abundance of individual DPs can be determined (Nakata & Troy, 2005). This approach avoids loss of long-chain polySia during DMB derivatisation, thereby improving the accuracy of maximum DP determination. Moreover, complete acid hydrolysis of fractionated polySia increases the sensitivity in detecting long-chain polySia (Drake et al., 2008). Using this approach, Nakata successfully detected polySia with approximately 400 Sia units from transfected Neuro2A cells (Nakata & Troy, 2005), while polySia containing more than 140 Sia residues was identified from human NK cells by Drake et al. (2008). However, the approach is restricted in use by the specificity of endo-β-galactosidase recognising the polySia-glycoprotein glycan linkage (Galuska, 2013) and might not be applicable for polySia with very long chain lengths, which is highly sensitive to pH and temperature, as discussed in Section 2.2.3.

3.3 Mass spectrometric strategies for structural analysis of polySia

Historically, mass spectrometric analysis of polySia has been a challenging task as the polymer is a mixture of indeterminate chain lengths with polycationic functionality. However, soft ionisation-based MS techniques have yielded progress on MS analysis of polySia, both to determine the mass composition and the degree of polymerisation of polySia derived from biological samples (Galuska, Geyer, Muhlenhoff, & Geyer, 2007; Kronewitter et al., 2014; Wang & Wang, 2019).

3.3.1 Structural analysis of polySia by MALDI-TOF MS

Matrix-assisted laser desorption/ionisation coupled with a time-of-flight mass analyser (MALDI-TOF) was the first MS approach used for polySia structural characterisation (Galuska et al., 2007). Analysis by MS is facilitated by polySia lactonisation (using mild acidic conditions) which neutralises the cationic charge of the internal Sia residues resulting in singly-charged polySia ions (**Fig. 6A, B**) for detection by MALDI. By setting distinct MALDI-TOF parameters, the exact mass composition and degree of polymerisation of polySia can both be achieved.

MALDI-TOF in negative reflectron mode is the best choice to investigate the mass composition of lactonised polySia in each DP, since this mode provides unambiguous mass information. As reported, lactonised and unlactonised polySia were easily separated by MALDI-TOF in negative reflectron mode (**Fig. 6C)** (Galuska et al., 2007). However, it has limitations since it can only detect polySia of DP up to 50 units (Galuska et al., 2007). In contrast, negative linear mode MALDI-TOF is the common choice for investigating the maximum DP of lactonised polySia from biological samples, although its mass accuracy is lower than that achieved in reflectron mode. As reported by Galuska (2007), polySia of DP up to 100, and 40 Sia units has been successfully detected from polymer standards and very small quantities (nanogram levels) of polysialylated proteins by MALDI-TOF MS in linear mode, respectively (Galuska et al., 2007).

Quite recently, to further improve mass accuracy and sensitivity in detecting long-chain polySia in linear mode MALDI-TOF, Wang & Wang developed an approach to dynamically optimise the instrument settings, by increasing the extraction delay in ionisation and various low mass cut-off

levels for the TOF detector to minimise the detector saturation caused by low-mass ions and improve the sensitivity in detecting high-mass polySia (Wang & Wang, 2019). Using this approach, the distribution of lactonised long-chain polySia (DP > 60) can be observed (**Fig. 6D**) and polySia containing up to 96 Sia units from *E.coli* was also detectable (Wang & Wang, 2019).

MALDI-TOF is also capable of distinguishing α-2,9-linked polySia from α-2,8-linked polySia, as both forms require distinctly different matrices to complete their lactonisation processes (Galuska et al., 2007).

Despite its potential, MALDI has poor tolerance to sample impurities, such as salts which often negatively impact results significantly. Electrospray ionisation (ESI), another soft ionisation technique, coupled with ion trap tandem mass spectrometric approaches, has also been useful in investigating polySia linkage and structure (Galuska et al., 2010).

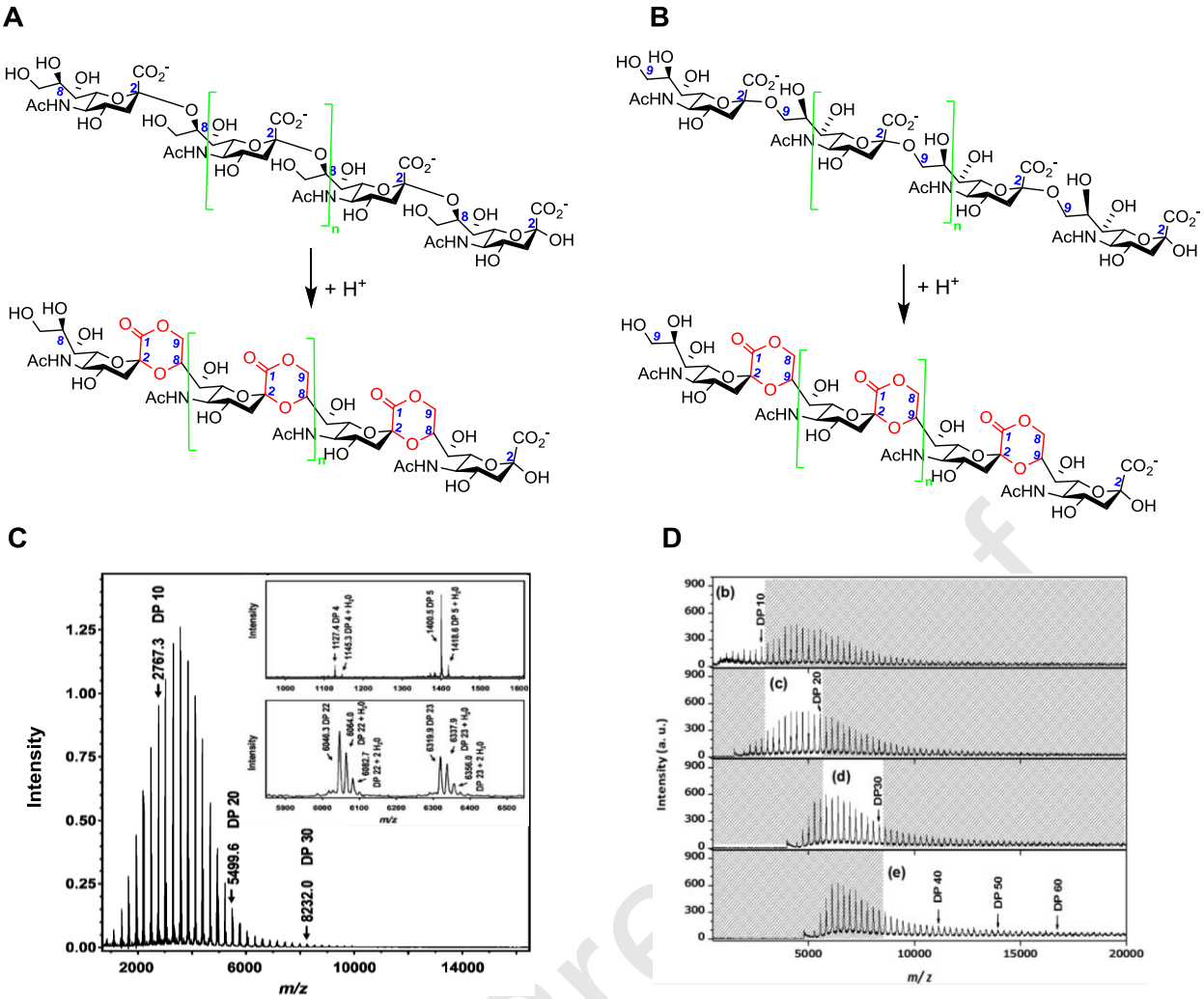


Fig. 6. Structural analysis of polySia polymers by MALDI-TOF. Lactonisation of (A) α-2,8-linked polySia, and (B) α-2,9-linked polySia, from colominic acids. (C) The exact mass composition of lactonised polySia is analysed by a MALDI-TOF in negative reflectron mode. Reproduced with permission from (Galuska et al., 2007), Copyright 2007, American Chemical Society. (D) to a MALDI-TOF/TOF in negative linear mode with various low-mass range cut-off; reproduced with permission from (Wang & Wang, 2019), Copyright 2018, Elsevier.

3.3.2 Structural analysis of polySia using an ESI-ion trap

Recently, the use of an Orbitrap MS (a high resolution ion trap mass analyser), coupled with a soft MS source, Sub-ambient Pressure Ionisation with Nanoelectrospray (SPIN) injection (Iglesias, 2015), has been applied to profiling lactonised polysialylated N-linked glycans (Kronewitter et al.,

2014). The injection technique provides a mild desolvation profile to minimise the loss of polySia caused by in-source fragmentation, thereby improving sensitivity (Kronewitter et al., 2014). Moreover, the Orbitrap approach greatly enhances mass resolution (Liu et al., 2014). By applying this SPIN-Orbitrap MS technique, glycans containing short-chain polySia (DP 7-11) have been detected in small quantities (100 μl) of human serum (Kronewitter et al., 2014). In addition, by coupling to nano-flow tandem chromatography, it allows for online sample de-salting and analysis of polySia of different chain lengths simultaneously in high resolution, within a single analysis. That said, this approach is less accessible due to the relatively very high cost of Orbitrap equipment, and the challenges of 'big data' analysis.

3.4 Immunochemical-based techniques for characterisation of polySia

Antibodies are important tools in detecting polySia in biological systems. In recent decades, a range of sensitive antibodies against polySia have been employed. In combination with specific sialidases, polySia can be characterised by antibodies in biological samples, without the need for sample preparation. A detailed evaluation of the utility and specificity of different anti-polySia antibodies and sialidases is described (Sato & Kitajima, 2008; Kitajima, Varki, & Sato, 2015). PolySia antibodies have proved useful in qualitative analysis but are limited by the threshold number of Sia units recognised, and are unable to distinguish polySia of different chain lengths above this threshold.

Table 1. Summary of studies in which polySia has been successfully detected in biological samples (1993 - 2019)

Samples	Year	PolySia identified	Maximal DP detected	PolySia-bioconjugate Extraction (if any)	PolySia-bioconjugate pre-treatment (if any)	Separation (if any)	Detection	Ref
Rainbow trout	1993	polyNeu5Gc	> 6	AEC & SEC fractionation	MH	Silica gel TLC	RD	(Sato et al., 1993)
Sea urchin eggs	1994	poly-Neu5Gc	> 7	SEC & Phenol extraction	MH	Silica gel TLC	RD	(Kitazume et al., 1994)
Rainbow trout	1995	poly-Neu5Gc	8	AEC & SEC fractionation	MH	Silica gel TLC	RD	(Sato et al., 1995)
Salmon eggs	1997	poly-Neu5Gc	11	Phenol extraction, WAE & SEC	MH	CarboPac PA-1	PAD	(Shimamura et al., 1983; Zhang et al., 1997)
Rainbow trout	1997	oligo-KDN	8	AEC & SEC	MH	CarboPac PA-1	PAD	(Zhang et al., 1997)
Sea urchin eggs	1999	poly-Neu5Gc	45-50	AEC & SEC	MH	CarboPac PA-100	PAD	(Lin et al., 1999)
Rainbow trout	1999	poly-Neu5Gc	10	AEC & SEC	DMB derivatisation	MonoQ HR 5/5	FD	(Lin et al., 1999)
Sea urchin eggs	1999	poly-Neu5Gc	20	AEC & SEC	DMB derivatisation	MonoQ HR 5/5	FD	(Lin et al., 1999)
Adult chick brains	2000	poly-α2,8-Neu5Ac	35	C/M extraction, ppt, SEC	MH	CarboPac PA-100	PAD	(Inoue et al., 2000)
Embryonic chick brain	2000	poly-α2,8-Neu5Ac	55	C/M extraction, ppt, SEC	MH	CarboPac PA-100	PAD	(Inoue et al., 2000)
Adult chick brains	2000	poly-α2,8-Neu5Ac	16	C/M extraction, ppt, SEC	DMB derivatisation	MonoQ column	FD	(Inoue et al., 2000)
Embryonic chick brain	2000	poly-α2,8-Neu5Ac	25	C/M extraction, ppt, SEC	DMB derivatisation	MonoQ column	FD	(Inoue et al., 2000)
Rat brains	2001	poly-α2,8-Neu5Ac	40-50	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Inoue & Inoue, 2001a)
Embryonic chick brain	2001	poly-α2,8-Neu5Ac	> 50	C/M extraction, ppt, SEC	DMB derivatisation	Mono-Q HR 10/10	FD	(Inoue & Inoue, 2001b)
IMR-32 cells	2002	poly-α2,8-Neu5Ac	> 40	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Poongodi et al., 2002)
PC12 cells	2002	poly-α2,8-Neu5Ac	> 40	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Poongodi et al., 2002)
Calf brain	2002	poly-α2,8-Neu5Ac	40	IAC	MH	CarboPac PA-100	PAD	(von Der Ohe et al., 2002) (Wuhrer et al., 2003)
Human milk	2003	poly-α2,8-Neu5Ac	18	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Yabe et al., 2003)
Embryonic chick brain	2003	poly-α2,8-Neu5Ac	60	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Inoue & Inoue, 2001a)
Murine NB41A3 cells	2003	poly-α2,9-Neu5Ac	> 30	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Inoue, Poongodi, Suresh, Jennings, & Inoue, 2003)
Neuro2A-ST8SIA4 cells	2004	poly-α2,8-Neu5Ac	~ 400	Immunoprecipitation	Endo-βG treatment HPAEC fractionation & CH	DNAPac PA-100/ TSK-gel ODS-120T	FD	(Nakata & Troy, 2005)
Sea urchin sperm (*H. p.*)	2004	poly-α2,9-Neu5Ac	16	C/M extraction, SEC & AEC	DMB derivatisation	Mono Q	FD	(Miyata et al., 2004; Miyata et al., 2006)
Sea urchin sperm (*H. p.*)	2004	poly-α2,9-Neu5Ac	> 7	C/M extraction, SEC & AEC	MH	Silica gel TLC	RD	(Miyata et al., 2004)
Sea urchin sperm (*H. p.*)	2004	poly-α2,9-Neu5Ac	20	C/M extraction, SEC & AEC	Mild alkaline $NaBH_4$ treatment	Mono Q	UV	(Miyata et al., 2004; Miyata et al., 2006)
Sea urchin sperm (*S. p.*)	2006	poly-α2,9-Neu5Ac	13-15	C/M extraction, SEC & AEC	Mild alkaline $NaBH_4$ treatment	Mono Q	UV	(Miyata et al., 2006)
Sea urchin sperm (*S. p.*)	2006	poly-α2,9-Neu5Ac	13	C/M extraction, SEC & AEC	MH	Mono Q	FD	(Miyata et al., 2006)
Sea urchin sperm	2006	poly-α2,9-Neu5Ac	15-20	C/M extraction, SEC & AEC	Mild alkaline $NaBH_4$ treatment	Mono Q	UV	(Miyata et al., 2006)
Sea urchin sperm	2006	poly-α2,9-Neu5Ac	12	C/M extraction, SEC & AEC	MH	Mono Q	FD	(Miyata et al., 2006)

Newborn mouse brain	2006	poly-α2,8-Neu5Ac	57	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Galuska et al., 2006)
Perinated mouse brain	2007	poly-α2,8-Neu5Ac	40	IAC	Lactonisation	n/a	MALDI-TOF	(Galuska et al., 2007)
N. meisseria meningitidis	2007	poly-α2,9-Neu5Ac	20	IAC	Lactonisation	n/a	MALDI-TOF	(Galuska et al., 2007)
Human NK cells	2008	poly-α2,8-Neu5Ac	> 140	Immunoprecipitation	Endo-βG treatment HPAEC fractionation + CH	DNAPac PA-100/ TSK-gel ODS-120T	FD	(Drake et al., 2008)
Mouse brain	2008	poly-α2,8-Neu5Ac	45	IAC	DMB derivatisation	DNAPac PA-100	FD	(Galuska et al., 2008)
Mouse brain	2008	poly-α2,8-Neu5Ac	~ 104	IAC	MH + HPAEC fractionation + CH	DNAPac PA-100	FD	(Galuska et al., 2008)
Zebrafish embryos	2009	poly-α2,8-Neu5Ac	7	C/M extraction	DMB derivatisation	Mono Q	FD	(Chang et al., 2009)
Healthy human lung	2013	poly-α2,8-Neu5Ac	45	IAC	DMB derivatisation	DNAPac PA-100	FD	(Ulm et al., 2013)
Human semen	2013	poly-α2,8-Neu5Ac	> 40	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Simon et al., 2013)
Embryonic pig brain	2014	poly-α2,8-Neu5Ac	41	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Nishimura et al., 2014)
Adult pig brain	2014	poly-α2,8-Neu5Ac	31	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Nishimura et al., 2014)
Human blood serum	2014	poly-α2,8-Neu5Ac	7-11	n/a	PNGase F treatment, SPE	Tandem nano flow rate HPLC	Orbitrap MS	(Kronewitter et al., 2014)
Roe deer testis	2014	poly-α2,8-Neu5Ac	> 17	IAC	DMB derivatisation	DNAPac PA-100	FD	(Hansch et al., 2014)
Feline ovaries	2015	poly-α2,8-Neu5Ac	>= 60	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Kaese et al., 2015)
N. meningitides cells	2016	Poly-Neu5Ac	30	ppt, AEC & UF	n/a	CarboPac PA-100	PAD	(Richard et al., 2016)
IMR-32 cells	2017	poly-α2,8-Neu5Ac	> 30	C/M extraction	DMB derivatisation	DNAPac PA-100	FD	(Abe et al., 2017)
Bovine cervical mucins	2017	poly-α2,8-Neu5Ac	50	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Galuska, Galuska, et al., 2017)
Human plasma	2018	poly-α2,8-Neu5Ac	> 40	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Zlatina et al., 2018)
Enzyme mixture	2018	poly-α2,8-Neu5Ac	25	n/a	n/a	Mono Q HR 5/5	UV	(Volkers et al., 2018)
E.coli	2019	poly-α2,8-Neu5Ac	96	n/a	Lactonisation	n/a	MALDI-TOF	(Wang & Wang, 2019)
Human serum	2019	poly-α2,8-Neu5Ac	> 30	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Kuhnle et al., 2019)
Human milk	2019	poly-α2,8-Neu5Ac	> 30	Dynabead enrichment	DMB derivatisation	DNAPac PA-100	FD	(Kuhnle et al., 2019)
E.coli	2019	poly-α2,8-Neu5Ac	70	UF, organic ppt, FPLC	DMB derivatisation	DNAPac PA-100	FD	(Bossmann et al., 2019)
E.coli	2019	poly-α2,8-Neu5Ac	130	UF, organic ppt, FPLC	n/a	DNAPac PA-100	CAD	(Bossmann et al., 2019)

Abbreviations:

AEC, anion exchange chromatography; SEC, size exchange chromatography; IAC, immunoaffinity chromatography; C/M, chloroform/ methanol; ppt, protein precipitation; UF, ultrafiltration; MH, mild acid hydrolysis; CH, complete hydrolysis; SPE, solid phase extraction; FPLC, fast protein liquid chromatography; TLC, thin layer chromatography; RD, resorcinol detection; PAD, pulsed amperometric detection; FD, fluorescence detection; UV, ultraviolet detection; CAD, charged aerosol detector; n/a, not applicable

4. Approaches for polySia quantification

Over recent decades, α-2,8-linked polySia has been extensively studied in mammalian tissues and cells. Expression levels are highly variable and tissue-specific. PolySia is usually expressed in high abundance in normal embryonic brains, with low abundance in normal adult brains (Seki & Arai, 1993; Angata et al., 1997; Ong et al., 1998). However, polySia is found to be re-expressed in a large number of cancer cell lines and malignant human tumours principally of neuroendocrine origin, notably neuroblastoma and small cell lung cancer (Finne, Finne, Deagostini-Bazin, & Goridis, 1983; Falconer et al., 2012). This abnormal expression of PolySia-NCAM in cancer is highly-correlated with disease progression (Falconer et al., 2012). Furthermore, polySia expression is also found on the surface of bacteria, where it is correlated with virulence of for example *Escherichia coli* K1 and group B *Neisseria meningitidis.* Therefore, quantitation of expression levels of polySia and polysialylated-proteins between normal tissues/cells vs diseased tissues/cells is vital, and can offer valuable insights into the biological roles of polySia in disease progression. This section discusses the techniques available for quantitative analysis of polySia and polysialylated-proteins from biological matrices.

4.1 Fluorometric HPLC quantitative analysis of polySia

Fluorometric HPLC analysis has proved well-suited to the measurement of the degree of polymerisation of polySia as discussed in Section 3.2.3. The approach can also be applied to quantification of polySia from biological matrices. Based on the final analytes, quantitative fluorometric analysis of polySia can be divided into two categories - the ‘direct’ method and the ‘indirect’ method. In the direct method, polySia is quantified directly through HPAEC-fluorometric analysis, while the indirect method quantifies derivatised hydrolysates of polySia.

4.1.1 HPAEC-FD quantitative analysis of polySia

Besides offering information on polySia DP, the HPAEC-FD approach can also provide quantitative analysis of polySia. Since each polySia chain is labelled with one fluorescent tag, the peak area associated with polySia in each DP is representative of the total number of polySia in that specific DP. Therefore, relative quantitation of polySia of a specific DP can be performed by comparing fluorescence peak areas between calibration standards and biological samples. Besides the advantage of providing quantitative analysis of polySia of specific DP, the HPAEC-FD approach can also determine quantitative differences in polySia DP distribution (Poongodi et al.,

2002; Nishimura et al., 2014; Abe et al., 2017). A DNAPac-PA100 column combined with a fluorescence detector was used to investigate the relative quantitative changes in expression of polySia chain lengths with time (24-84 h) in IMR-32 (human neuroblastoma) and PC-12 (rat pheochromocytoma) cancer cells (Poongodi et al. 2002). This approach was also used to compare the expression levels of polySia containing more than 6 Sia units from embryonic and adult pig brains, and found the relative quantitative result was in good agreement with that obtained from an ELISA study utilising anti-polySia antibodies (Nishimura et al., 2014). Furthermore, changes in cellular expression levels of polySia (DP > 6) in response to drug treatment have been described (Abe et al., 2017). However, HPAEC-FD has relatively poor sensitivity in the measurement of longer chain polySia, probably due to the de-polymerisation during fluorescent derivatisation (as described in Section 3). Another disadvantage is that derivatisation of polySia requires lengthy reaction times (up to 24 h), albeit at low temperature (4 °C), to minimise the partial hydrolysis of long-chain polySia, as discussed earlier (Poongodi et al., 2002; Nishimura et al., 2014; Abe et al., 2017).

4.1.2 Fractionation-based RPLC-FD quantitative analysis of polySia hydrolysate

To avoid partial hydrolysis of long-chain polySia during derivatisation, an indirect fluorometric quantitative polySia analysis method based on fractionation and strong acid hydrolysis of polySia is described. In this approach, the liberated polySia is fractionated according to its DP by HPAEC and subsequently the polySia fractions are hydrolysed into sialic acid monomers under strong acidic conditions. Following fluorescence derivatisation, the sialic acid monomers are analysed by RPLC-FD. (Nakata & Troy, 2005). The relative quantitation of polySia possessing different DPs can be achieved by comparing changes in fluorescence peak areas derived from corresponding sialic acid hydrolysates following RPLC-FD analysis. The key merit of this approach is the accurate quantitative information of long chain polySia (Galuska, 2013). Moreover, acid hydrolysis of long chain polySia into Sia monomers significantly increases detection sensitivity. However, this approach has limited application due to its dependence on liberation of polySia from glycoproteins by endo-β-galactosidase, which requires specific linkage recognition on the N-glycan core of polySia (Nakata & Troy, 2005; Al-Saraireh et al., 2013; Galuska, 2013; Gnanapragassam et al., 2014), and polySia with very long chain lengths might still be sensitive to the pH and temperature used in the enzyme reaction, as described previously.

4.1.3 Periodate fluorometric C7/C9 (sialic acid) analysis of polySia

Approaches based on periodate oxidation of sialic acid, with subsequent fluorometric analysis using RPLC-FD, are an indirect quantitative method for polySia analysis. It provides highly sensitive and selective analysis of internal Sia residues of oligo- and polySia from glycoproteins,

by oxidation of the *terminal* Sia residues (Sato, Inoue, Matsuda, & Kitajima, 1998; Miyata et al., 2004; Hane, Sumida, Kitajima, & Sato, 2012; Simon et al., 2013; Nishimura et al., 2014; Galuska, Galuska, et al., 2017). In the presence of sodium periodate, the vicinal diols in the C7, C8 and C9 positions of Sia (**Fig. 7A**) are oxidised to yield a seven carbon 'C7-aldehyde' (Sato et al., 1998; Galuska, 2013; Kitajima et al., 2015). Borohydride reduction of the C7-aldehyde to a 'C7-hydroxy-Sia' results in a 'C7-Sia'. Fluorescently labelled C9-Sia can be separated from the C7-Sia and quantitated using RPLC-FD due to polarity differences (Sato et al., 1998). Since sialic acid residues in polySia are linked via α-2,8 glycosidic bonds, the C8 position cannot be periodate-oxidised. This means only the terminal Sia residues at the non-reducing end of a polySia chain are amenable to periodate oxidation, while the internal Sia residues remain unaffected by this treatment (Sato et al., 1998; Inoue & Inoue, 2003; Galuska, 2013). Therefore, the internal Sia residues can be distinguished by RPLC-FD after fluorescence derivatisation, (**Fig. 7B**), and the percentage of the total internal Sia residues over that of terminal Sia can be evaluated by comparison with the corresponding fluorescence peak areas of C7 and C9 Sia (Sato et al., 1998). The advantage of this approach is the high sensitivity and specificity demonstrated by detection of low (ng) amounts of internal sialic acid residues of oligo- and polySia molecules (Sato et al., 1998). Another advantage of C7/C9 Sia analysis in the study of polySia is the mild reaction conditions required for periodate oxidation. Although pH 5.5 is optimal for periodate oxidation, the reaction also can proceed at physiological pH and at low temperatures (4 °C) (Stevenson, Huang, Studdert, & Hartley, 2004). The downside of the approach in quantitative analysis is the risk of underestimating the amount of short-chain polySia (low DP), as the number of the oxidised terminal Sia forms a higher percentage in polySia of low DP than that of high DP. Also, it cannot provide information as to the precise number of polySia chains, and might lead to false positive quantitative results for internal Sia residues of polySia in biological samples (Nishimura et al., 2014). Moreover, the oxidation of α-2,9-linked polySia has also been reported using this method (Inoue & Inoue, 2003). It also is noteworthy that permethylation of Sia followed by NMR analysis is an alternative approach for unambiguous detection of α-2,8- and α-2,9-linked polySia (Finne, Krusius, Rauvala, & Hemminki, 1977; Galuska, 2013; Yamakawa et al., 2018; Komura et al., 2019).

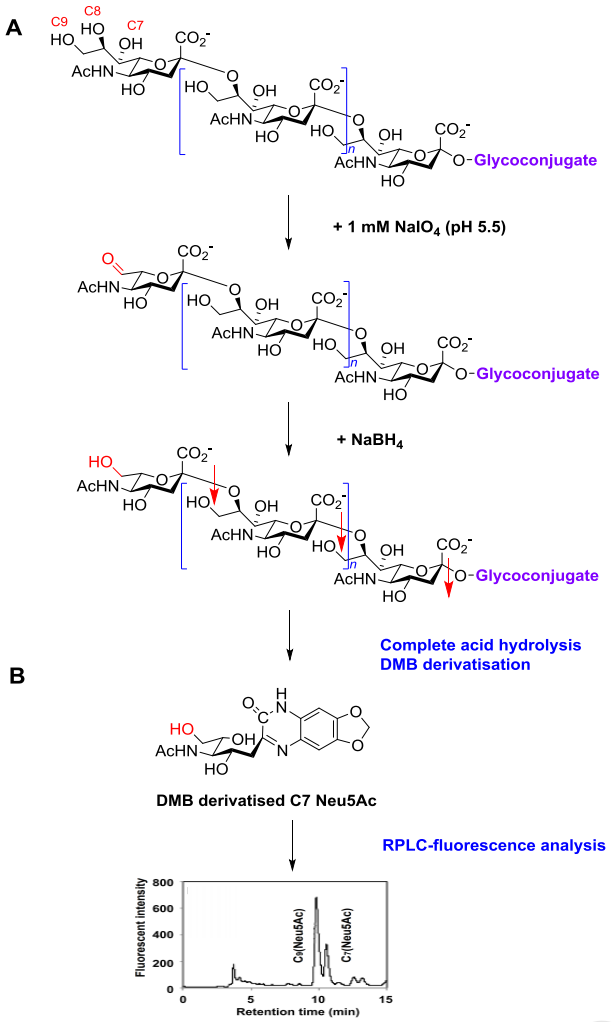


Fig. 7. Workflow of C7/C9 analysis of polySia - by periodate oxidation followed by DMB derivatisation. (A) PolySia is periodate oxidised and borohydride reduced to create a 7-carbon Sia derivative; **(B)** After acid hydrolysis, the DMB-derivatised C7 Sia can be separated from the C9 Sia using reversed-phased chromatography. Chromatogram reprinted with permission from (Nishimura et al., 2014), Copyright, 2014, Omics Publishing Group.

4.1.4 HPAEC-CAD quantitative analysis of polySia

In very recent studies describing the HPAEC-coupled CAD detection of polySia, the Beutel group also developed and validated an HPAEC-CAD method for the quantitative analysis of free polySia polymers. In this approach, the oligo-Sia and short-chain polySia were first removed by a low gradient of salts in HPAEC chromatography, followed by the quick elution of all polySia polymers containing more than 20 Sia units by high salt-containing buffers (Bossmann et al., 2019). Interestingly, the calibrated relationship between CAD signal and polySia concentration was fitted in a semi-linear equation at low concentrations and a polynomial function at high concentrations. The lowest amount of purified polySia from biological sources for quantitative

calibration was 250 ng (Bossmann et al., 2019). As described in section 3.2.4, the key virtue of this approach is to avoid the time-consuming fluorescent derivatisation and the partial hydrolysis of long-chain polySia. The second advantage of this quantitative approach is that it allows rapid quantification of total polySia. As discussed in 3.2.4, this method is limited in resolving long-chain polySia, hence it might not serve the quantitative requirements to analyse polySia of specific chain length.

4.2 Quantitative Immunodetection

Immunodetection is widely-used as sensitive tools in the study of expression levels of polysialylated-proteins in clinical samples. The details of polySia-specific antibodies have been summarised by Sato and Kitajima (Sato & Kitajima, 2008; Kitajima et al., 2015). The most commonly-used immunoassays for quantitative analysis of polySia and polysialylated-proteins in biological matrices are antibodies to enable detection and quantitation by enzyme-linked immunosorbent assay (ELISA), Western blotting and Flow cytometry.

4.2.1 Enzyme-linked immunosorbent assay (ELISA)

Among the available immunochemical methods, ELISA is the most straightforward available to study polySia and polysialylated-proteins from biological mixtures. Indirect ELISA has often been used for polySia quantification. This utilises an anti-polySia antibody as the primary (capture) antibody to detect polySia in cells and tissues, coupled with a secondary antibody (detection and quantitation antibody) (Dubois, Okandze, Figarella-Branger, Rampini, & Rougon, 1995; Sato et al., 1995; Hildebrandt et al., 1998; Poongodi et al., 2002; Seidenfaden, Krauter, Schertzinger, Gerardy-Schahn, & Hildebrandt, 2003; Amoureux et al., 2010). ELISA is generally a reproducible and reliable method providing high specificity and sensitivity. Sandwich ELISAs, which employ two antibodies to recognise two different epitopes of target polysialylated proteins can also be used. (Dubois et al., 1995; Amoureux et al., 2010; Piras et al., 2015). For example, as little as 600 pg of polySia-NCAM per microgram of brain extract was determined using a Sandwich ELISA employing an anti-Meningococcus group B antibody as the capture antibody (Dubois et al., 1995). Also determined by this approach was a comparison of polySia-NCAM expression in brain normal and tumour tissue. Expression of polySia-NCAM in the healthy adult human brain was 8 pg of polysialylated-NCAM per µg of total proteins, while levels in patients with medulloblastoma tumours significantly increased to 150-1030 pg per µg of total protein (Amoureux et al., 2010). Besides the high sensitivity and specificity, ELISA techniques enable the

assessment of polySia from very small samples (Dubois et al., 1995). However, wherever antibodies are used, the technique is reliant upon their specificity, as previously discussed. Another disadvantage of ELISA is that it cannot distinguish polySia of different chain lengths (Amoureux et al., 2010). Nevertheless, ELISA is still a sound choice for rapid and accurate quantitation of total polysialylated-proteins in biological samples, given the relatively wide availability of very reliable and specific anti-polySia antibodies.

4.2.2 Western blotting (semi-quantitative)

Western blotting is a widely-used technique for evaluating relative expression levels of polysialylated-proteins in biological samples (von Der Ohe et al., 2002; Korja et al., 2009; Valentiner, Muhlenhoff, Lehmann, Hildebrandt, & Schumacher, 2011; Nishimura et al., 2014; Westphal, Kleene, Lutz, Theis, & Schachner, 2016; Abe et al., 2017; Chen, Ren, Zhang, Troy, & Wang, 2017; Klobucar et al., 2017). Similarly to ELISA, Western blotting also recognises polySia proteins *via* immunodetection. Here, protein expression levels are quantified based on the differential densitometry of the associated chemiluminescent and/or fluorescent signals from the blots. The major merit of Western blotting in quantitative analysis is its high specificity, as it allows separation of polysialylated-proteins by size and is less dependent on antibody specificity compared to ELISA. However, it is difficult to accurately quantify polysialylated-proteins in Western blotting, as bands of polysialylated-proteins are usually smeared due to the wide range of masses as a result of the multiple various polySia chain lengths on the polySia-conjugated proteins. Moreover, Nishimura et al found that the relative quantitative result was underestimated in Western blotting compared to ELISA when using mAb 735 anti-polySia antibody (Nishimura et al., 2014). This was suggested to be due to the low blotting efficiency in PVDF membranes of long chain polySia compared to short chain polySia, since the mAb 735 antibody can only recognise internal sialyl residues of polySia (Nishimura et al., 2014). In summary, Western blotting is more suitable for qualitative and semi-quantitative analysis of total polysialylated-proteins in biological samples rather than accurate absolute quantitative analysis.

4.2.3 Flow cytometry in the analysis of cell surface polySia expression

Flow cytometry allows rapid and quantitative evaluation of polySia expression on the surface of intact cells based using fluorescence-labelled polySia recognising probes, such as anti-polySia antibodies and Inactive-Endosialidase-Green Fluorescent (fusion) Protein (iEndoN-GFP), developed by the Finne group (Jokilammi, Korja, Jakobsson, & Finne, 2007). As with other immunoassays, flow cytometry relies on specific immunological or enzymatic recognition. The

expression levels of cell surface polySia can be evaluated by the detected fluorescence intensity. The method is advantageous in offering rapid information as to quantitative changes in cell surface polySia expression, and can be high throughput. Furthermore, the use of iEndoN-GFP in Flow cytometry allows the number of polySia positive cells in a cell population to be determined.

5. Conclusion

This review demonstrates that a wide range of analytical techniques has been used for structural and quantitative analysis of polySia present in biological samples, and that sample preparation strategies can add greatly to the sensitivity and selectivity of analysis, as summarised in **Fig. 2 and Fig. 8**. The techniques available vary in sample treatment, analysis protocols and the types of information they provide. Sample treatments that involve chemical modification are at the forefront of structural characterisation of polySia. HPAEC-FD of hydrolysed and fluorescent labelled polySia is the most popular method for structural analysis, due to its high selectivity, sensitivity, superior polymer separation and relative high tolerance to the complexity of biological samples. On the other hand, improvements in mass spectrometry-based approaches now offer more informative structural details and can be used complementary to HPAEC-FD. These analytical methods benefit greatly from inclusion of immunoaffinity-based sample clean-up to provide high specificity and efficiency in polySia extraction.

Immunoassays still represent the most frequently reported method for rapid quantitation or semi-quantitation of polySia and polysialylated proteins from complex biological matrices. Western blotting is most widely used for semi-quantitative analysis of polysialylated proteins, while ELISA allows the absolute quantitation of total polySia and/or polysialylated proteins. Alternatively, quantitative analysis of polySia can also be achieved by chemical C7/C9 and HPAEC based fractionation strategies. Both immunoassays and C7/C9 approaches are the preferred choice for rapid quantitative analysis of total polySia, while the HPAEC based approaches are not only the choice for quantitation of total polySia but also available to quantify polySia of each DP, owing to the efficient separation of polySia at high resolution.

There is a growing interest in polySia due to its roles in development, and diseases such as cancer and microbial infection. Functional characterisation of polySia in each DP group, especially when at low abundance, means that new methods for quantitative analysis of polySia in clinical samples are urgently required. However, due to the limited quantities of material often available from clinical samples, chemical modification/fluorescence derivatisation may not be sufficient to provide quantitative information for polySia of each DP group. Furthermore, the issue of chemical degradation of polySia through these methodologies remains problematic, despite efforts to minimise this. PolySia antibodies or inactive-EndoN for detection cannot detect the absolute abundance of each group of polySia. Taken together, these shortcomings currently deprive us of the ability to obtain a truly accurate measure of cellular polySia expression. While significant progress has been made, the development of more rapid, highly-sensitive techniques to conduct accurate quantitative analysis when challenged by low polySia abundance, without affecting

polySia chemical structure integrity, and to quantify of each polySia DP group remain important goals for the future.

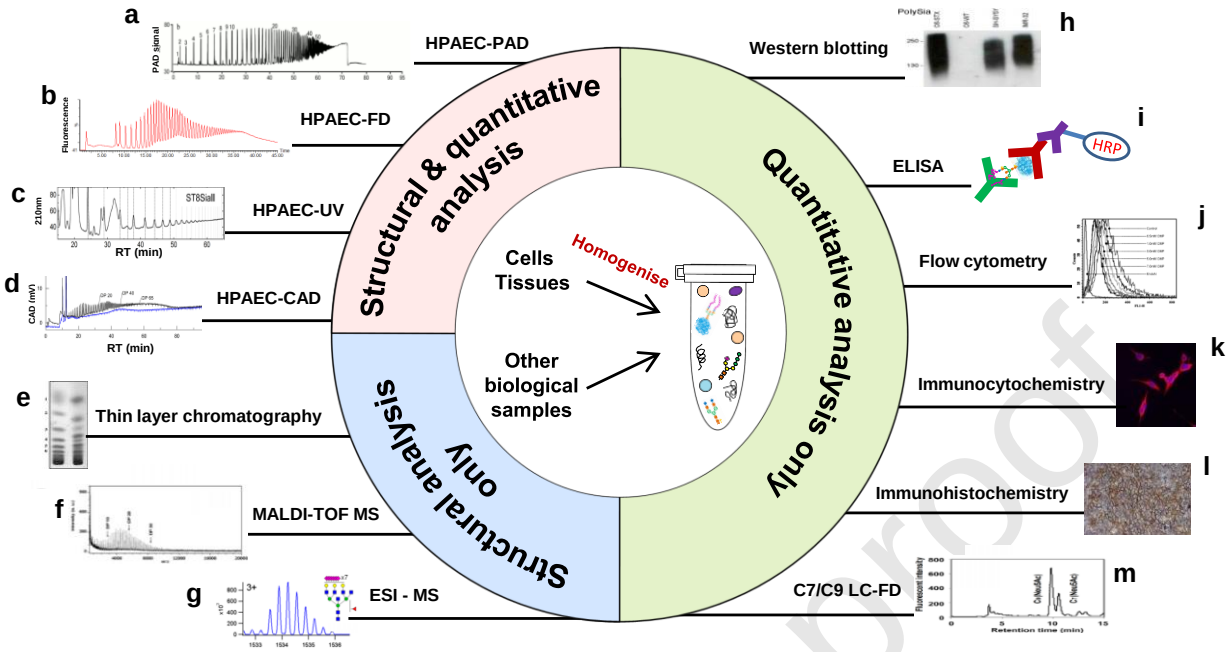


Fig. 8. Summary of strategies of analysing polySia and their bioconjugates in biological matrices. (a) Reprinted with permission from (Rohrer, Basumallick, & Hurum, 2013), Copyright 2013, Pleiades Publishing; (c) Reprinted with permission from (Volkers et al., 2018), Copyright 2018, American Chemical Society; (d) Reprinted with permission from (Bossmann et al., 2019), Copyright 2019, Elsevier; (e) Reprinted with permission from (Miyata et al., 2004), Copyright 2004, Oxford University Press; (f) Reprinted with permission from (Wang & Wang, 2019), Copyright 2018, Elsevier; (g) Reprinted with permission from (Kronewitter et al., 2014), Copyright 2014, American Chemical Society; (m) Reprinted with permission from (Nishimura et al., 2014), Copyright, 2014, Omics Publishing Group.

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