^{19}F labelled Glycosaminoglycan Probes for Solution NMR and Non-linear (CARS) Microscopy

Marcelo A. Lima[1,2], Renan P. Cavalheiro[1], Gustavo M.Viana[1], Maria C.Z. Meneghetti[1], Timothy R. Rudd[3,2], Mark A. Skidmore[4], Andrew K. Powell[5,2] and Edwin A. Yates[2,1,4*]

1. *Department of Biochemistry, UNIFESP, Rua Três de Maio, Vila Clementino, São Paulo, 40440-SP Brazil*
2. *Department of Biochemistry, University of Liverpool, Liverpool L69 7ZB United Kingdom*
3. *The National Institute of Biological Standards and Controls, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG United Kingdom*
4. *School of Life Sciences, Keele University, Staffordshire ST5 5BG United Kingdom*
5. *School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF United Kingdom*

**Correspondence to eayates@liv.ac.uk*

Abstract

Studying polysaccharide-protein interactions under physiological conditions by conventional techniques is challenging. Ideally, macromolecules could be followed by both *in vitro* spectroscopy experiments as well as in tissues using microscopy, to enable a proper comparison of results over these different scales but, often, this is not feasible. The cell surface and extracellular matrix polysaccharides, glycosaminoglycans (GAGs) lack groups that can be detected selectively in the biological milieu. The introduction of ^{19}F labels into GAG polysaccharides is explored and the interaction of a labelled GAG with the heparin-binding protein, antithrombin, employing ^{19}F NMR spectroscopy is followed. Furthermore, the ability of ^{19}F labelled GAGs to be imaged using CARS microscopy is demonstrated. ^{19}F labelled GAGs enable both ^{19}F NMR protein-GAG binding studies in solution at the molecular level and non-linear microscopy at a microscopic scale to be conducted on the same material, essentially free of background signals.

Key words: Heparin, NMR, ^{19}F, Non-linear microscopy

Introduction

An understanding of the biological processes that determine cell-cell signalling, hence coordinate cellular responses and maintain healthy growth and development, must encompass a detailed appreciation of interactions between proteins and extracellular matrix (ECM) polysaccharides [1-3]. Moreover, the network of interactions that defines healthy homeostasis is also relevant to disease processes, many of which involve some modification to this network [1]. One practical challenge is how to study phenomena, particularly interactions, that correlate with natural or disease processes, but which range in scale from the dimensions of molecules to the macroscopic level of tissues and organisms. Current approaches at the molecular level include spectroscopic and crystallographic techniques, which allow investigation at the level of ensembles of molecules, while various forms of (mainly) optical microscopy allow tissues to be examined in detail at the macroscopic level. However, few techniques or tools have the ability to work across these scales. Each approach also tends to employ distinct and largely incompatible labelling procedures and detection techniques, hence, it can be difficult to interpret them together, or extrapolate results from one technique to the other and, consequently, to relate the observations to the biological processes under investigation. Clearly, it would be advantageous if it were possible to utilise the same materials throughout.

Studying interactions between proteins and polysaccharides in solution presents a number of further challenges. There are several reasons why conventional techniques for the detailed study of proteins, either in the solid state employing crystallography, or in solution using NMR, are often unsuitable when the protein to be studied is bound to a polysaccharide ligand. First, it is not usually possible to crystallise a protein in the presence of a polysaccharide and, especially in the case of extracellular polysaccharides such as the glycosaminoglycans (GAGs), the polysaccharide is frequently a complex and heterogeneous mixture of sequences and chain dimensions. The problem has been managed in several crystallographic and NMR studies by employing short oligosaccharide fragments acting as proxies for their parent polysaccharide and this has revealed some structural details of their interactions [4-7]. The use of oligosaccharide fragments as a stand-in for the polysaccharide is widespread, although there are differences in binding properties which have their origins in the distinct conformational and motional behaviour of oligo- compared to polysaccharides but, possibly, also in the different numbers of potential binding sites in the two cases [8]. In the case of ^{1}H and ^{13}C NMR, the size and low mobility of protein-polysaccharide complexes in solution can lead to line broadening. Additional techniques with which to follow the interactions between proteins and GAGs are therefore required. One possibility is solid state NMR, which has been used to obtain detailed information in aggregates consisting of peptides and GAGs [9,10] but, it would also be desirable to be able to follow the location of GAGs in tissues by microscopy. One family of emerging techniques, based on Raman Spectroscopy, involves the selective observation of chemical groups that are particular to the molecules of interest. Unfortunately, there are no obvious Raman signals which are unique to GAGs. Their characteristic carboxylate, amine, acetyl and sulfate groups are also present to some extent in other biological molecules. With these considerations in mind, we have sought a label which has desirable characteristics both in terms of sensitivity in microscopy and NMR, exhibits very low or no background signal in biological systems and that can be informative as regards changes in its environment. The ^{19}F nucleus fulfils many of these criteria and here, we investigate the possibility of employing ^{19}F labelling of GAG poly- and oligosaccharides as a route to unambiguous information concerning protein-polysaccharide interactions using NMR spectroscopy, and as a potential means of following events in tissues employing non-linear (CARS) microscopy.

The ^{19}F nucleus constitutes 100% of the fluorine occurring naturally and, with nuclear spin ½, presents readily-interpretable NMR signals, which are sensitive to the surrounding chemical environment owing to the lone pair electrons in the outer shell of the ^{19}F atom. A strong paramagnetic component dominates the chemical shift and, usefully, also provides good signal dispersion. ^{19}F NMR spectra in biological systems are very clean since almost no ^{19}F is present in biology naturally [11] and is particularly useful in systems whose size, immobility and/or complexity precludes conventional NMR approaches, such as transmembrane proteins and macromolecular complexes. The sensitivity of the ^{19}F nucleus to its immediate environment, including to solvent water or deuterium oxide (in D_2O compared to D_2O/H_2O 80:20, v/v, $\Delta\delta$ = 0.13 ppm), also makes it suitable for studies involving site-specific labelling, denaturation experiments [12] and for identifying interactions at protein interfaces. In addition, the temperature dependence of ^{19}F NMR signal line widths renders it sensitive to mobility, from which information relating to both binding and stability can be deduced. Although not investigated here, the chemistry employed to introduce ^{19}F into biomolecules is also suitable for ^{18}F, opening-up the possibility of conducting positron emission tomography (PET). Labelling of proteins with ^{18}F [13], as well as of sugars during their chemical synthesis has been reported [14,15] and the chemical labelling of amino acid side chains with fluorine via a range of amino acid side chains (aliphatic, aromatic and cysteine) has also been achieved [16]. Such labels can have effects on the structure of the protein however, and one approach that has been explored to minimise this problem is to conduct partial substitution [17,18].

The attachment of suitable ^{19}F containing groups to glycosaminoglycan (GAG) polysaccharides would extend many of the advantages exploited for proteins to the GAG class of molecules, thereby opening-up the study of polysaccharide-protein interactions. The choice of label made here; *N*-trifluoroacetyl, which is readily introduced to free amino groups of glucosamine residues within the polysaccharide chains, and trifluoroalkylamines, (either 2,2,2-trifluoroethylamine (TFEA) or 3,3,3 trifluoropropylamine (TFPA)), which can be introduced through amide formation onto the former carboxylate groups of uronic acids via the 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC)-activated ester, both contain a terminal -CF_3 group. These provide a clean signal in ^{19}F NMR, owing to short T_1 relaxation times and low chemical shift anisotropy but, usefully, also provide distinct Raman transitions, exploitable in coherent Anti-Stokes Raman Scattering (CARS) and other, non-linear microscopy techniques. Signals arising from C-F bond stretching are also unambiguous, there being effectively no background signals. Labelling GAG macromolecules with ^{19}F using -CF_3 groups can therefore provide both a suitable NMR signal with favourable spectroscopic properties capable of providing information regarding molecular interactions *in vitro,* but also in tissues [12], as well as a signal that can be used in non-linear microscopy (such as CARS), which is free of background and interference from water, allowing direct chemical imaging. Two straightforward methods of introducing ^{19}F into GAG polysaccharides (denoted (i) and (ii) below) are reported. Illustrative examples of their use as probes of molecular interactions in solution using ^{19}F NMR spectroscopy and the ability to image them using CARS microscopy are demonstrated.

Methods

*(i) Preparation of trifluoroacetylated glycosaminoglycan polysaccharides (**1**):-* The first procedure is based on *O*-acylation using acetic anhydride, in which iodine has been proposed to act as a Lewis acid catalyst [19]. The free amino groups of glucosamine residues in heparin (used widely as an experimental proxy for the more scarce, naturally occurring ligand, heparan sulfate) polysaccharide derivatives, in which free amino groups had been introduced [20], was labelled selectively using trifluoroacetic anhydride with iodine as catalyst. With this class of large, anionic polysaccharides, however, the reaction was found to be highly selective for the free amino groups of the glucosamine residues of the polysaccharides, forming trifluoroacetamido-derivatives. De-N-sulfated heparin polysaccharide (25 mg, ~40 µmol of disaccharide equivalents) was added as a solid to trifluoroacetic anhydride (9.5 mmol), with solid iodine flakes (0.2 mmol) and stirred at room temperature. The reactants were then precipitated in ice-cold ethanol (200 mL) and filtered, washed with ethanol, any large iodine grains were removed and the filtrate was washed until any remaining iodine had been removed (brown colour subsides: *n.b*. iodine in the wash was neutralised by the addition of solid sodium metabisulfite until a clear iodide containing solution had been obtained and was then disposed of). The recovered GAG compounds; *N*-trifluoroacetyl heparin (**1**) was dialysed (7 kDa cut-off dialysis membrane (SpectraPore, USA)) 3 times against 2 L of distilled water, the dialysate was recovered, then subjected to gel permeation chromatography (Sephadex G-25) and characterised by ^{19}F [**Table 1**] and ^{13}C NMR spectroscopy [**Supp. Fig. 1**] prior to being employed in experiments.

*(ii) Introduction of trifluoroalkyl groups at carboxylate groups of uronic acids of heparin and the heparin-derived pentasaccharide, Arixtra™, via EDC activation (**2**), (**3**) and (**4**):-* The second method involved attachment of the ^{19}F label via the carboxylate groups of uronate residues in GAGs. The carboxylate groups were activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Pearce) in 50 mM HEPES buffer and the resulting EDC ester reacted with an alkylamine; either 2,2,2 trifluoethylamine (TFEA) or 3,3,3 trifluoropropylamine (TFPA), both convenient water soluble fluoroamines, of moderate volatility (b.p. 36-37 and 67.5-68 °C respectively), to form the corresponding fluoroamides. [*Note*: - If EDC is added before the

alkylamine to the GAG solution, a side-product persists, evident in ^{13}C and ^{1}H NMR, thought to originate from a rearrangement of the isourea adduct [21]. This provides an alternative labelling method, acting specifically on the carboxylate groups of the uronic acids. The method was applied to both GAG polysaccharides (described in (a) below) and to the pentasaccharide antithrombotic drug Arixtra™, whose systematic name, [α-D-Glucopyranoside, methyl *O*-2- deoxy-6-*O*-sulfo-2-(sulfoamino) -α-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranuronosyl-(1→4)-*O*-2-deoxy-3,6-di-O-sulfo -2-(sulfoamino)-α-D-glucopyranosyl-(1→4)- *O*-2-*O*-sulfo-α-L- idopyranuronosyl- (1→4) -2-deoxy-2-(sulfoamino)-6-(hydrogen sulfate), sodium salt], is abbreviated to 'AGAIA', and described in (b).

*(a). Derivatisation of Ido-2-de-sulfated heparin and heparin with TFEA (**2**) and (**3**)*:- The ido-2-de-O-sulfated porcine mucosal heparin[20] (for the preparation of (**2**)) or porcine mucosal heparin (for the preparation of (**3**)) (25 mg) was dissolved in 50 mM HEPES buffer (0.50 mL, pH 6.8), TFEA (61 µmol) added, followed by EDC (26 µmol). The reaction mixture was then stirred at room temperature (30 mins) and the products ((**2**) or (**3**)) were dialysed (7 kDa cut-off dialysis membrane (SpectraPore, USA)) 3 times against 2 L of distilled water, the dialysate recovered, subjected to gel permeation chromatography (Sephadex G-25) and employed in experiments.

*(b). Derivatisation of AGAIA pentasaccharide with TFPA (**4**)*:- The pentasaccharide, AGAIA (Arixtra™) (2.5. mg, 1.7 umol in 0.5 mL 137 mM NaCl) was mixed with HEPES buffer (0.5 mL, 50 mM, pH 6.8) and TFPA (2 µL, 22 µmol) were added, followed by EDC (1 mg, 5.2 µmol) and shaken for 10 mins at room temperature. The products were then desalted by gel permeation chromatography using Sephadex G-25, freeze dried and characterised by ^{19}F NMR (**Table 1**) before being employed in binding experiments.

(iii). *^{19}F NMR investigation of GAG interaction with antithrombin in solution*:- Decoupled ^{19}F NMR experiments were conducted on a Bruker Avance III 500 MHz spectrometer (UNICAMP, Instituto de Quimica, Campinas, Brazil) using antithrombin (AT; 100 µL, 15 mg/mL in PBS containing D_2O) and additions of (**4**), (500 µL, 1 mg/mL) or heparin (500 µL, 5 mg/mL) [**Fig. 1**].

(iv). *Selective Detection of ^{19}F labelled heparin-derived polysaccharides using Non-linear (CARS) Microscopy:-* The form of Raman microscopy used here is based on a non-linear Raman technique termed coherent anti-Stokes Raman scattering (CARS). This technique can generate coherent signals up to 10^5 times stronger than conventional Raman and relies on the use of light as a pump to alter the populations of vibrational states, then to probe, providing anti-Stokes light emission of higher energy (i.e. lower wavelength) than that of the pump [22]. The ^{19}F labelled heparin derivatives (**1**) to (**3**) were used in CARS Raman microscopy experiments, in which the Raman signal from the CF_3 group provided a means of detecting selectively a film of the polysaccharide by means of its CARS signal [**Fig. 2**].

Results and Discussion

The fluorinated products were first characterised by ^{19}F NMR to provide a range of complementary probes, suitable for GAG-protein interactions studies and non-linear spectroscopy [**Table 1**]. The reaction was also applied to the antithrombin (AT) binding pentasaccharide, (AGAIA) and was found, surprisingly, to label selectively the GlcA carboxylate group [**Supp. Fig. 2**]. This product was then shown by ^{19}F NMR spectroscopy to bind AT in solution [**Fig. 1A;** lower panel (unbound) and middle panel (bound)] by virtue of a change in chemical shift position downfield of unreacted TFPA and binding was confirmed using fluorescence shift assay [**Fig. 1B**].

The binding of (**4**) to AT in solution was then disrupted by the addition of excess unlabelled heparin [**Fig. 1A;** upper panel]. Furthermore, the ^{19}F labelled AGAIA pentasaccharide was able to stabilise AT to an extent comparable to the unmodified form [**Fig. 1B**] confirming its interaction with antithrombin [23].

Images (1.16 mm x 1.16 mm) were recorded in the forward CARS (F-CARS) mode on a Leica TSC-SP8 CARS instrument (Wetzler, Germany), employing pump lasers (the first pump laser selected at 928.2 nm and the second CARS laser, fixed, at 1064.5 nm) both at 46 % power. Images, detected at 1379 cm^{-1} pre-determined to offer useful C-F derived Raman signals [23] were recorded at 0.05 frames/s with 1.20 µs dwell time per pixel, a gain of 850.82 V and at 10 x magnification. Five µL of compounds (**1**), (**2**) and (**3**) were dried from solutions (1 mg/mL in H_2O) on a glass slide and imaged [**Fig. 2A**, **2B** and **2C**]. As a control, the second laser (1064.5 nm) was then switched-off and no image was observed, confirming that the images were CARS-derived (**Fig. 2D**).

Both methods of labelling heparin and other GAG derivatives are suitable for conducting NMR and microscopy studies and this will allow the same reagent to be used to examine events on scales ranging from those of molecules to tissues, providing more readily interpretable data. There are many other types of selective labelling that could be exploited to allow ^{19}F NMR and non-linear microscopy with other classes of macromolecules, such as proteins. One example would be to incorporate ^{19}F labelled Trp residues, which can be achieved biosynthetically through either 5- or 6- fluorouridine. In this way, it will also be possible to follow proteins in tissues and the GAG polysaccharides with which they interact, as well as to study exactly the same interactions *in vitro* using ^{19}F NMR. One interesting finding was that, in contrast to the situation observed for heparin polysaccharides, the reaction of TFPA with the pentasaccharide, AGAIA, did provide highly selective reaction with the carboxylate group of the GlcA residue over those of IdoA [**Supp. Fig. 2**] perhaps reflecting its more accessible, equatorial conformation. The use of CARS microscopy to study molecular interactions in tissues is in its infancy but, promises to be able to investigate complex assemblies by virtue of its ability to selectively observe a range of chemical groups, whether they be naturally occurring, or have been introduced deliberately. This paper proposes a number of ^{19}F-labelled GAG probes which can also be used in both NMR and CARS microscopy applications.

Acknowledgements

MAL and EAY gratefully acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support. The authors also gratefully acknowledge Dr Tony Curtis of Keele University for the provision of ^{19}F NMR spectra. Dr Andrew V. Stachulski of the Department of Chemistry, University of Liverpool is thanked for useful discussions and advice. The authors also thank Dr. Claudio Tormena of University of Campinas (UNICAMP), Brazil for provision of NMR facilities.

References

1. Ori, A., Wilkinson, M.C., Fernig, D.G.: A systems biology approach for the investigation of the heparin/heparan sulfate interactome. The Journal of biological chemistry **286**(22), 19892-19904 (2011). doi:10.1074/jbc.M111.228114
2. Solari, V., Borriello, L., Turcatel, G., Shimada, H., Sposto, R., Fernandez, G.E., Asgharzadeh, S., Yates, E.A., Turnbull, J.E., DeClerck, Y.A.: MYCN-dependent expression of sulfatase-2 regulates neuroblastoma cell survival. Cancer research **74**(21), 5999-6009 (2014). doi:10.1158/0008-5472.CAN-13-2513
3. Xu, R., Rudd, T.R., Hughes, A.J., Siligardi, G., Fernig, D.G., Yates, E.A.: Analysis of the fibroblast growth factor receptor (FGFR) signalling network with heparin as coreceptor: evidence for the expansion of the core FGFR signalling network. The FEBS journal **280**(10), 2260-2270 (2013). doi:10.1111/febs.12201
4. Guerrini, M., Elli, S., Mourier, P., Rudd, T.R., Gaudesi, D., Casu, B., Boudier, C., Torri, G., Viskov, C.: An unusual antithrombin-binding heparin octasaccharide with an additional 3-O-sulfated glucosamine in the active pentasaccharide sequence. The Biochemical journal **449**(2), 343-351 (2013). doi:10.1042/BJ20121309
5. Guglier, S., Hricovini, M., Raman, R., Polito, L., Torri, G., Casu, B., Sasisekharan, R., Guerrini, M.: Minimum FGF2 binding structural requirements of heparin and heparan sulfate oligosaccharides as determined by NMR spectroscopy. Biochemistry **47**(52), 13862-13869 (2008).
6. Viskov, C., Elli, S., Urso, E., Gaudesi, D., Mourier, P., Herman, F., Boudier, C., Casu, B., Torri, G., Guerrini, M.: Heparin dodecasaccharide containing two antithrombin-binding pentasaccharides: structural features and biological properties. The Journal of biological chemistry **288**(36), 25895-25907 (2013). doi:10.1074/jbc.M113.485268
7. Wei, Z., Deakin, J.A., Blaum, B.S., Uhrin, D., Gallagher, J.T., Lyon, M.: Preparation of heparin/heparan sulfate oligosaccharides with internal N-unsubstituted glucosamine residues for functional studies. Glycoconjugate journal **28**(8-9), 525-535 (2011). doi:10.1007/s10719-011-9352-3
8. Powell, A.K., Yates, E.A., Fernig, D.G., Turnbull, J.E.: Interactions of heparin/heparan sulfate with proteins: appraisal of structural factors and experimental approaches. Glycobiology **14**(4), 17R-30R (2004). doi:10.1093/glycob/cwh051
9. Madine, J., Clayton, J.C., Yates, E.A., Middleton, D.A.: Exploiting a (13)C-labelled heparin analogue for in situ solid-state NMR investigations of peptide-glycan interactions within amyloid fibrils. Organic & biomolecular chemistry **7**(11), 2414-2420 (2009). doi:10.1039/b820808e
10. Madine, J., Pandya, M.J., Hicks, M.R., Rodger, A., Yates, E.A., Radford, S.E., Middleton, D.A.: Site-specific identification of an abeta fibril-heparin interaction site by using solid-state NMR spectroscopy. Angewandte Chemie **51**(52), 13140-13143 (2012). doi:10.1002/anie.201204459
11. Danielson, M.A., Falke, J.J.: Use of 19F NMR to probe protein structure and conformational changes. Annual review of biophysics and biomolecular structure **25**, 163-195 (1996). doi:10.1146/annurev.bb.25.060196.001115
12. Feeney, J., McCormick, J.E., Bauer, C.J., Birdsall, B., Moody, C.M., Starkmann, B.A., Young, D.W., Francis, P., Havlin, R.H., Arnold, W.D., Oldfield, E.: 19F Nuclear Magnetic Resonance Chemical Shifts of Fluorine Containing Aliphatic Amino Acids in Proteins: Studies on Lactobacillus casei Dihydrofolate Reductase Containing (2S,4S)-5-Fluoroleucine. Journal of the American Chemical Society **118**(36), 8700-8706 (1996). doi:10.1021/ja960465i
13. Chang, Y.S., Jeong, J.M., Lee, Y.S., Kim, H.W., Rai, G.B., Lee, S.J., Lee, D.S., Chung, J.K., Lee, M.C.: Preparation of 18F-human serum albumin: a simple and efficient protein labeling

method with 18F using a hydrazone-formation method. Bioconjugate chemistry **16**(5), 1329-1333 (2005). doi:10.1021/bc050086r
14. Boutureira, O., D'Hooge, F., Fernandez-Gonzalez, M., Bernardes, G.J., Sanchez-Navarro, M., Koeppe, J.R., Davis, B.G.: Fluoroglycoproteins: ready chemical site-selective incorporation of fluorosugars into proteins. Chemical communications **46**(43), 8142-8144 (2010). doi:10.1039/c0cc01576h
15. Boutureira, O., Rodriguez, M.A., Diaz, Y., Matheu, M.I., Castillon, S.: Studies on the Zn(II)-mediated electrophilic selenocyclization and elimination of 3,4-O-isopropylidene-protected hydroxyalkenyl sulfides: synthesis of a 2-phenylselenenyl glycal. Carbohydrate research **345**(8), 1041-1045 (2010). doi:10.1016/j.carres.2010.03.001
16. Klein-Seetharaman, J., Getmanova, E.V., Loewen, M.C., Reeves, P.J., Khorana, H.G.: NMR spectroscopy in studies of light-induced structural changes in mammalian rhodopsin: applicability of solution (19)F NMR. Proceedings of the National Academy of Sciences of the United States of America **96**(24), 13744-13749 (1999).
17. Kitevski-LeBlanc, J.L., Evanics, F., Scott Prosser, R.: Optimizing (1)(9)F NMR protein spectroscopy by fractional biosynthetic labeling. Journal of biomolecular NMR **48**(2), 113-121 (2010). doi:10.1007/s10858-010-9443-7
18. Kitevski-Leblanc, J.L., Hoang, J., Thach, W., Larda, S.T., Prosser, R.S.: (1)(9)F NMR studies of a desolvated near-native protein folding intermediate. Biochemistry **52**(34), 5780-5789 (2013). doi:10.1021/bi4010057
19. Ravindranathan, A., Parks, T.N., Rao, M.S.: New isoforms of the chick glutamate receptor subunit GluR4: molecular cloning, regional expression and developmental analysis. Brain research. Molecular brain research **50**(1-2), 143-153 (1997).
20. Yates, E.A., Santini, F., Guerrini, M., Naggi, A., Torri, G., Casu, B.: 1H and 13C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. Carbohydrate research **294**, 15-27 (1996).
21. 3.5 Carbodiimides. In: Felix, A., Moroder, L., Toniolo, C. (eds.) Houben-Weyl Methods of Organic Chemistry Vol. E 22a, 4th Edition Supplement, vol. E 22 a. Methoden der Organischen Chemie (Houben-Weyl). Georg Thieme Verlag, Stuttgart, (2004)
22. Maker, P.D., Terhune, R.W.: Study of Optical Effects Due to an Induced Polarization Third Order in the Electric Field Strength. Physical Review **137**(3A), A801-A818 (1965).
23. Chaffin, J.C.T., Marshall, T.L.: Using a gas cell to characterize FT-IR air sensor performance. In: 1999, pp. 69-78

Figures and Tables

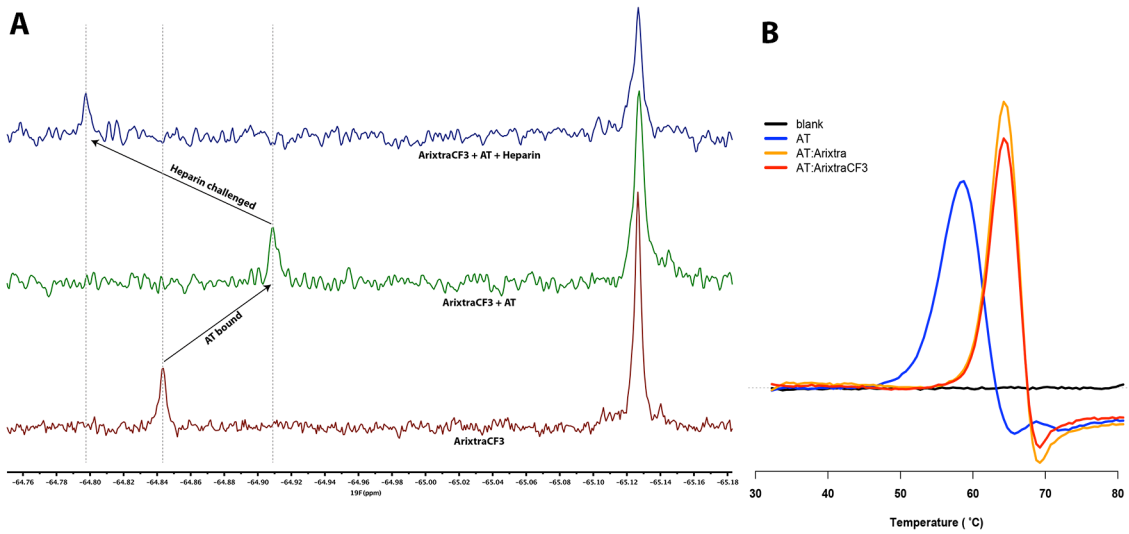


Figure 1. ^{19}F labelled GAGs can be used as probes of protein binding and of solution environment. ^{19}F NMR spectra of: **A**. (lower) ^{19}F-labelled AGAIA pentasaccharide (**4**) alone, (middle) bound ^{19}F-labelled AGAIA pentasaccharide in the presence of antithrombin (AT) and ^{19}F-labelled AGAIA pentasaccharide in the presence of antithrombin plus added heparin to compete-off the ligand, (upper) returning to a distinct chemical shift position, demonstrating the sensitivity of the ^{19}F label to the solution environment. **B**. Differential scanning fluorimetry showing that the ^{19}F labelled AGAIA pentasaccharide (**4**) stabilised AT (red curve, compared to AT alone, blue curve) to an extent comparable to the unlabelled pentasaccharide (gold curve).

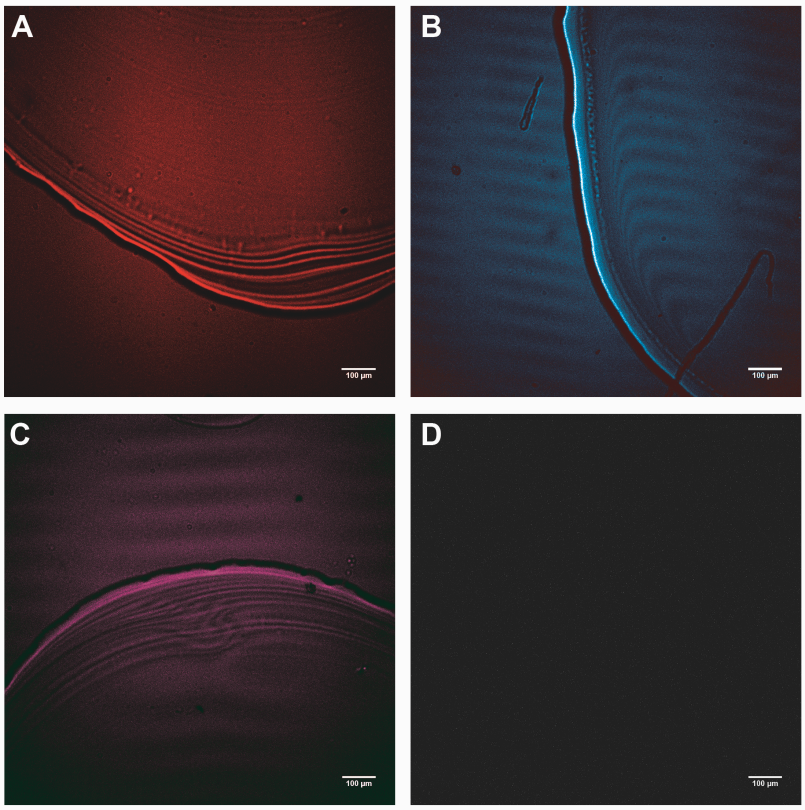


Figure 2. CARS images of dried films of ^{19}F labelled GAG (heparin) derivatives. A. Compound (**1**), **B**. Compound (**2**), **C**. Compound (**3**), **D**. Control experiment with the 1064.5 nm CARS laser switched-off, showing no image and confirming that the images in **A**-**C** are derived from CARS. Images were recorded at 10 x magnification and correspond to a field size of 1.16 mm x 1.16 mm.

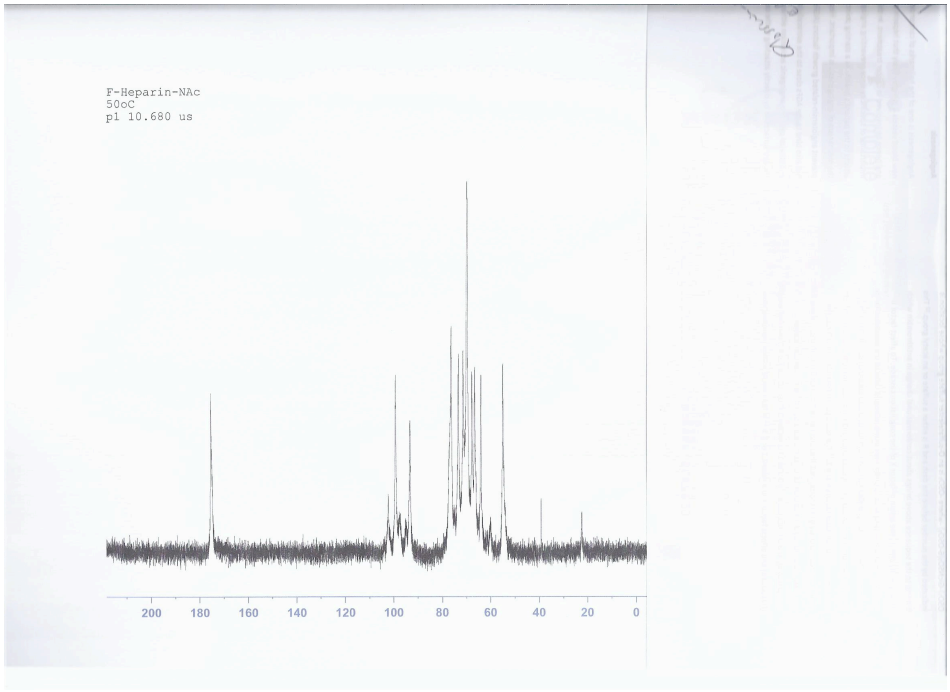
Table 1. ^{19}F NMR chemical shifts for (**1**) to (**4**).

Name	Compound	δ ^{19}F /ppm
N-Trifluoroacetyl porcine mucosal heparin	(**1**)	-75.58[a]
Trifluorethylamido-Ido-2-de-*O*-sulfated heparin	(**2**)	-72.2[a]
Trifluorethylamido-heparin	(**3**)	-72.3[a]
AGAIA (Arixtra™ pentasaccharide)	(**4**)	-64.8[b]

[a.] Recorded at 298 K on a 500 MHz Bruker Avance III HD NMR spectrometer with 5-mm BBO probe. Chemical shift values reported relative to TFA in D_2O at (δ ^{19}F), -75.61ppm. The chemical shift of (**1**) was also measured for a range of temperatures from 288 to 333 K, showing good linearity (Data not shown).

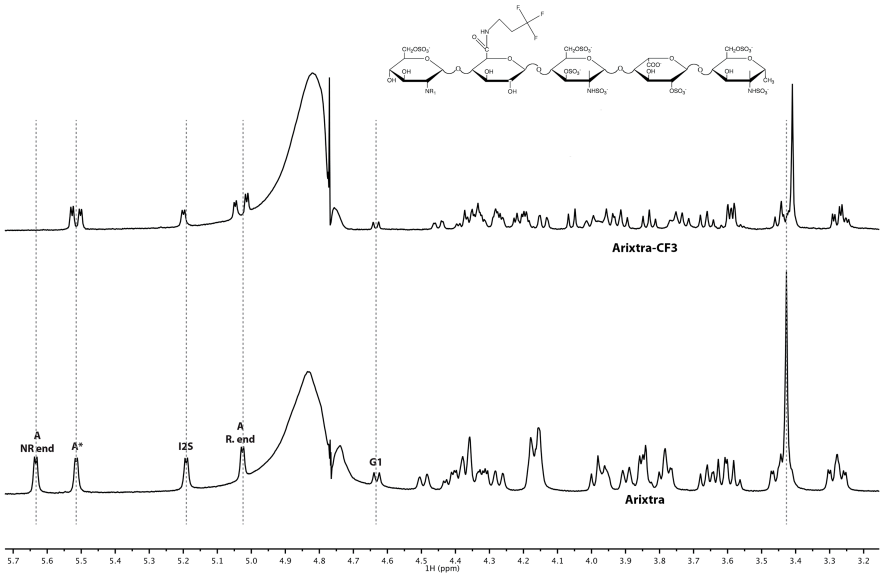
[b.] Recorded at 300 K relative to trifluoroethylamine (TFEA) at (δ ^{19}F), -65.12 ppm.

Supplementary Figure 1



Supp. Fig. 1. ^{13}C NMR spectrum of *N*-trifluoracetyl heparin (**1**) at 50 C in D_2O/H_2O

Supplementary Figure 2



Supp. Fig. 2. ^{1}H NMR spectra of *AGAIA* (**bottom**) and AGAIA-CF3 (**top**) at 30 ºC in D_2O