



LJMU Research Online

Lima, M, Cavaleiro, RP, Viana, G, Meneghetti, M, Rudd, TR, Skidmore, MA, Powell, AK and Yates, EA

19F labelled glycosaminoglycan probes for solution NMR and non-linear (CARS) microscopy

<http://researchonline.ljmu.ac.uk/id/eprint/4103/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Lima, M, Cavaleiro, RP, Viana, G, Meneghetti, M, Rudd, TR, Skidmore, MA, Powell, AK and Yates, EA (2016) 19F labelled glycosaminoglycan probes for solution NMR and non-linear (CARS) microscopy. Glycoconjugate Journal. ISSN 1573-4986

LJMU has developed [LJMU Research Online](#) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

[Click here to view linked References](#)

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

Marcelo A. Lima^{1,2}, Renan P. Cavalheiro¹, Gustavo M. Viana¹, Maria C.Z. Meneghetti¹, Timothy R. Rudd^{3,2}, Mark A. Skidmore⁴, 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, 04440-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 eyates@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 ¹⁹F labels into GAG polysaccharides is explored and the interaction of a labelled GAG with the heparin-binding protein, antithrombin, employing ¹⁹F NMR spectroscopy is followed. Furthermore, the ability of ¹⁹F labelled GAGs to be imaged using CARS microscopy is demonstrated. ¹⁹F labelled GAGs enable both ¹⁹F 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, ¹⁹F, 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.

1 Studying interactions between proteins and polysaccharides in solution presents a number
2 of further challenges. There are several reasons why conventional techniques for the detailed
3 study of proteins, either in the solid state employing crystallography, or in solution using NMR, are
4 often unsuitable when the protein to be studied is bound to a polysaccharide ligand. First, it is not
5 usually possible to crystallise a protein in the presence of a polysaccharide and, especially in the
6 case of extracellular polysaccharides such as the glycosaminoglycans (GAGs), the polysaccharide
7 is frequently a complex and heterogeneous mixture of sequences and chain dimensions. The
8 problem has been managed in several crystallographic and NMR studies by employing short
9 oligosaccharide fragments acting as proxies for their parent polysaccharide and this has revealed
10 some structural details of their interactions [4-9]. The use of oligosaccharide fragments as a stand-
11 in for the polysaccharide is widespread, although there are differences in binding properties which
12 have their origins in the distinct conformational and motional behaviour of oligo- compared to
13 polysaccharides but, possibly, also in the different numbers of potential binding sites in the two
14 cases [10]. In the case of ^1H and ^{13}C NMR, the size and low mobility of protein-polysaccharide
15 complexes in solution can lead to line broadening. Additional techniques with which to follow the
16 interactions between proteins and GAGs are therefore required. One possibility is solid state NMR,
17 which has been used to obtain detailed information in aggregates consisting of peptides and GAGs
18 [11,12] but, it would also be desirable to be able to follow the location of GAGs in tissues by
19 microscopy. One family of emerging techniques, based on Raman Spectroscopy, involves the
20 selective observation of chemical groups that are particular to the molecules of interest.
21 Unfortunately, there are no obvious Raman signals which are unique to GAGs. Their characteristic
22 carboxylate, amine, acetyl and sulfate groups are also present to some extent in other biological
23 molecules. With these considerations in mind, we have sought a label which has desirable
24 characteristics both in terms of sensitivity in microscopy and NMR, exhibits very low or no
25 background signal in biological systems and that can be informative as regards changes in its
26 environment. The ^{19}F nucleus fulfils many of these criteria and here, we investigate the possibility
27 of employing ^{19}F labelling of GAG poly- and oligosaccharides as a route to unambiguous
28 information concerning protein-polysaccharide interactions using NMR spectroscopy, and as a
29 potential means of following events in tissues employing non-linear (CARS) microscopy.
30
31
32
33
34
35

36 The ^{19}F nucleus constitutes 100% of the fluorine occurring naturally and, with nuclear spin
37 $\frac{1}{2}$, presents readily-interpretable NMR signals, which are sensitive to the surrounding chemical
38 environment owing to the lone pair electrons in the outer shell of the ^{19}F atom. A strong
39 paramagnetic component dominates the chemical shift and, usefully, also provides good signal
40 dispersion. ^{19}F NMR spectra in biological systems are very clean since almost no ^{19}F is present in
41 biology naturally [13] and is particularly useful in systems whose size, immobility and/or complexity
42 precludes conventional NMR approaches, such as transmembrane proteins and macromolecular
43 complexes. The sensitivity of the ^{19}F nucleus to its immediate environment, including to solvent
44 water or deuterium oxide (in D_2O compared to $\text{D}_2\text{O}/\text{H}_2\text{O}$ 80:20, v/v, $\Delta\delta = 0.13$ ppm), also makes it
45 suitable for studies involving site-specific labelling, denaturation experiments [14] and for
46 identifying interactions at protein interfaces. In addition, the temperature dependence of ^{19}F NMR
47 signal line widths renders it sensitive to mobility, from which information relating to both binding
48 and stability can be deduced. Although not investigated here, the chemistry employed to introduce
49 ^{19}F into biomolecules is also suitable for ^{18}F , opening-up the possibility of conducting positron
50 emission tomography (PET). Labelling of proteins with ^{18}F [15], as well as of sugars during their
51 chemical synthesis has been reported [16,17] and the chemical labelling of amino acid side chains
52 with fluorine via a range of amino acid side chains (aliphatic, aromatic and cysteine) has also been
53 achieved [18]. Such labels can have effects on the structure of the protein however, and one
54 approach that has been explored to minimise this problem is to conduct partial substitution [19,20].
55
56
57
58
59
60
61
62
63
64
65

1 The attachment of suitable ¹⁹F containing groups to glycosaminoglycan (GAG)
2 polysaccharides would extend many of the advantages exploited for proteins to the GAG class of
3 molecules, thereby opening-up the study of polysaccharide-protein interactions. The choice of label
4 made here; *N*-trifluoroacetyl, which is readily introduced to free amino groups of glucosamine
5 residues within the polysaccharide chains, and trifluoroalkylamines, (either 2,2,2-
6 trifluoroethylamine (TFEA) or 3,3,3 trifluoropropylamine (TFPA)), which can be introduced through
7 amide formation onto the former carboxylate groups of uronic acids via the 1-ethyl-3-(3-
8 dimethylaminopropyl) carbodiimide (EDC)-activated ester, both contain a terminal -CF₃ group.
9 These provide a clean signal in ¹⁹F NMR, owing to short T₁ relaxation times and low chemical shift
10 anisotropy but, usefully, also provide distinct Raman transitions, exploitable in coherent Anti-
11 Stokes Raman Scattering (CARS) and other, non-linear microscopy techniques. Signals arising
12 from C-F bond stretching are also unambiguous, there being effectively no background signals.
13 Labelling GAG macromolecules with ¹⁹F using -CF₃ groups can therefore provide both a suitable
14 NMR signal with favourable spectroscopic properties capable of providing information regarding
15 molecular interactions *in vitro*, but also in tissues [14], as well as a signal that can be used in non-
16 linear microscopy (such as CARS), which is free of background and interference from water,
17 allowing direct chemical imaging. Two straightforward methods of introducing ¹⁹F into GAG
18 polysaccharides (denoted (i) and (ii) below) are reported. Illustrative examples of their use as
19 probes of molecular interactions in solution using ¹⁹F NMR spectroscopy and the ability to image
20 them using CARS microscopy are demonstrated.
21
22
23
24

25 Methods

27 (i) Preparation of trifluoroacetylated glycosaminoglycan polysaccharides (1):- The first procedure is
28 based on *O*-acylation using acetic anhydride, in which iodine has been proposed to act as a Lewis
29 acid catalyst [21]. The free amino groups of glucosamine residues in heparin (used widely as an
30 experimental proxy for the more scarce, naturally occurring ligand, heparan sulfate) polysaccharide
31 derivatives, in which free amino groups had been introduced [22], was labelled selectively using
32 trifluoroacetic anhydride with iodine as catalyst. With this class of large, anionic polysaccharides,
33 however, the reaction was found to be highly selective for the free amino groups of the
34 glucosamine residues of the polysaccharides, forming trifluoroacetamido-derivatives. De-N-
35 sulfated heparin polysaccharide (25 mg, ~40 μmol of disaccharide equivalents) was added as a
36 solid to trifluoroacetic anhydride (9.5 mmol), with solid iodine flakes (0.2 mmol) and stirred at room
37 temperature. The reactants were then precipitated in ice-cold ethanol (200 mL) and filtered,
38 washed with ethanol, any large iodine grains were removed and the filtrate was washed until any
39 remaining iodine had been removed (brown colour subsides: *n.b.* iodine in the wash was
40 neutralised by the addition of solid sodium metabisulfite until a clear iodide containing solution had
41 been obtained and was then disposed of). The recovered GAG compounds; *N*-trifluoroacetyl
42 heparin (1) was dialysed (7 kDa cut-off dialysis membrane (SpectraPore, USA)) 3 times against 2
43 L of distilled water, the dialysate was recovered, then subjected to gel permeation chromatography
44 (Sephadex G-25) and characterised by ¹⁹F [Table 1] and ¹³C NMR spectroscopy [Supp. Fig. 1]
45 prior to being employed in experiments.
46
47
48
49
50
51
52

53 (ii) Introduction of trifluoroalkyl groups at carboxylate groups of uronic acids of heparin and the
54 heparin-derived pentasaccharide, Arixtra™, via EDC activation (2), (3) and (4):- The second
55 method involved attachment of the ¹⁹F label via the carboxylate groups of uronate residues in
56 GAGs. The carboxylate groups were activated using 1-ethyl-3-(3-dimethylaminopropyl)
57 carbodiimide (EDC, Pearce) in 50 mM HEPES buffer and the resulting EDC ester reacted with an
58 alkylamine; either 2,2,2 trifluoroethylamine (TFEA) or 3,3,3 trifluoropropylamine (TFPA), both
59 convenient water soluble fluoroamines, of moderate volatility (b.p. 36-37 and 67.5-68 °C
60 respectively), to form the corresponding fluoroamides. [Note: - If EDC is added before the
61
62
63
64
65

alkylamine to the GAG solution, a side-product persists, evident in ^{13}C and ^1H NMR, thought to originate from a rearrangement of the isourea adduct [23]. 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 ArixtraTM, whose systematic name, [α -D-Glucopyranoside, methyl O-2-deoxy-6-O-sulfo-2-(sulfoamino) - α -D-glucopyranosyl-(1 \rightarrow 4)-O- β -D-glucopyranuronosyl-(1 \rightarrow 4)-O-2-deoxy-3,6-di-O-sulfo -2-(sulfoamino)- α -D-glucopyranosyl-(1 \rightarrow 4)- O-2-O-sulfo- α -L- idopyranuronosyl- (1 \rightarrow 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 heparin²⁰ (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 (ArixtraTM) (2.5. mg, 1.7 μmol 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 [24]. 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**].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

The binding of (4) to AT in solution was then disrupted by the addition of excess unlabelled heparin [Fig. 1A; upper panel]. Furthermore, the ¹⁹F 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 [25] 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 H₂O) 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 ¹⁹F NMR and non-linear microscopy with other classes of macromolecules, such as proteins. One example would be to incorporate ¹⁹F 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 ¹⁹F 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 ¹⁹F-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 ¹⁹F 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. Pellegrini, L., Burke, D.F., von Delft, F., Mulloy, B., Blundell, T.L.: Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* **407**(6807), 1029-1034 (2000). doi:10.1038/35039551
9. Schlessinger, J., Plotnikov, A.N., Ibrahimi, O.A., Eliseenkova, A.V., Yeh, B.K., Yayon, A., Linhardt, R.J., Mohammadi, M.: Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Molecular cell* **6**(3), 743-750 (2000).
10. 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
11. 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
12. 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
13. 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

14. 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
15. 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
16. 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
17. 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 glycol. *Carbohydrate research* **345**(8), 1041-1045 (2010). doi:10.1016/j.carres.2010.03.001
18. 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 (¹⁹F) NMR. *Proceedings of the National Academy of Sciences of the United States of America* **96**(24), 13744-13749 (1999).
19. 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
20. 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
21. 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).
22. 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).
23. 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)
24. 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).
25. 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

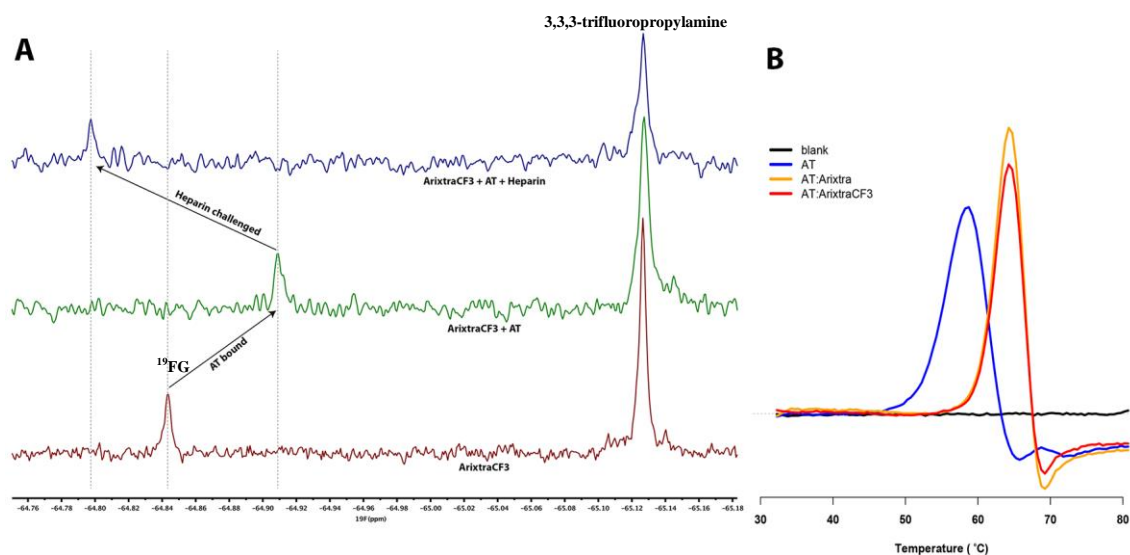


Figure 1. ^{19}F labelled GAGs can be used as probes of protein binding and of solution environment. ^{19}F NMR spectra of: **A.** (lower) ^{19}F -labelled AGAIA pentasaccharide (**4**) alone, (middle) bound ^{19}F -labelled AGAIA pentasaccharide in the presence of antithrombin (AT) and ^{19}F -labelled 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). ^{19}F G; 3,3,3-trifluoropropylamine labelled AGAIA on glucuronic acid.

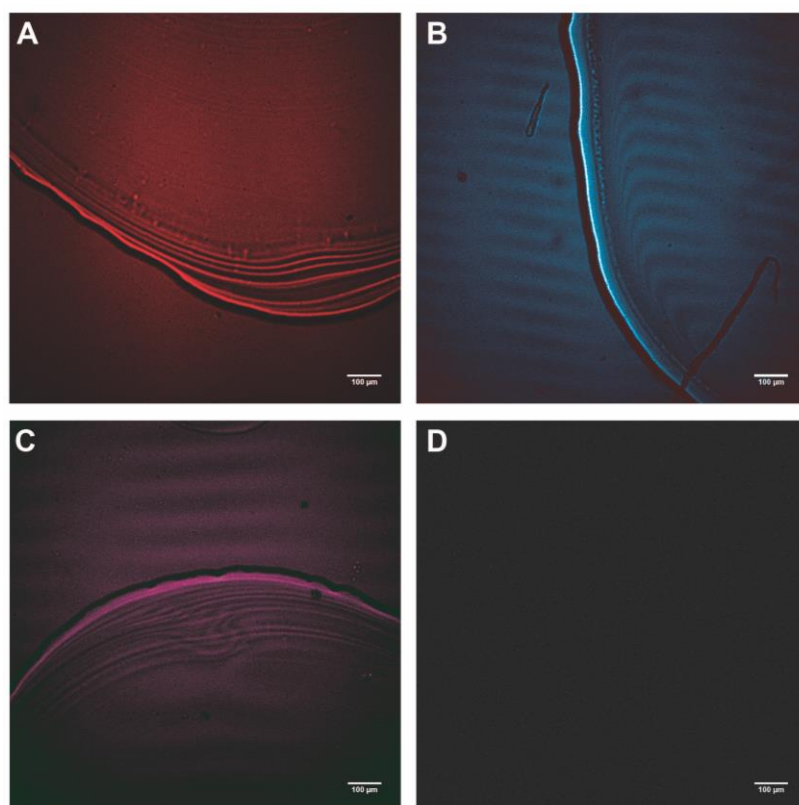


Figure 2. CARS images of dried films of ^{19}F labelled GAG (heparin) derivatives. A. Compound (1), **B.** Compound (2), **C.** Compound (3), **D.** Control experiment with the 1064.5 nm CARS laser switched-off, showing no image and confirming that the images in **A-C** are derived from CARS. Images 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	$\delta^{19}\text{F}$ /ppm
<i>N</i> -Trifluoroacetyl porcine mucosal heparin	(1)	-75.58 ^a
Trifluoroethylamido-Ido-2-de- <i>O</i> -sulfated heparin	(2)	-72.2 ^a
Trifluoroethylamido-heparin	(3)	-72.3 ^a
AGAIA (Arixtra™ pentasaccharide)	(4)	-64.8 ^b

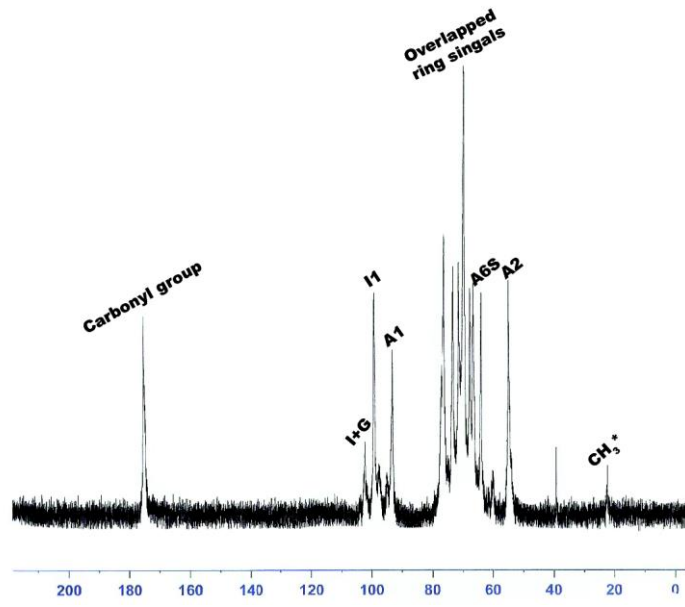
^a Recorded at 298 K on a 500 MHz Bruker Avance III HD NMR spectrometer with 5-mm BBO probe. Chemical shift values reported relative to TFA in D_2O at ($\delta^{19}\text{F}$), -75.61 ppm. The chemical shift of (1) was also measured for a range of temperatures from 288 to 333 K, showing good linearity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

^b Recorded at 300 K relative to trifluoroethylamine (TFEA) at ($\delta^{19}\text{F}$), -65.12 ppm.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

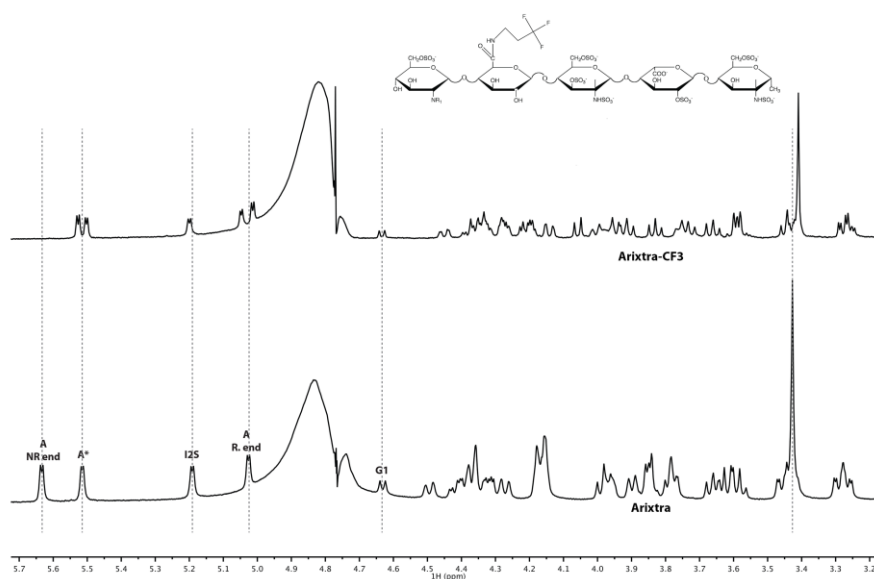
Supplementary Figure 1

Heparin ¹⁹FNAc



Supp. Fig. 1. ¹³C NMR spectrum of *N*-trifluoroacetyl heparin (**1**) at 50° C in D₂O/H₂O. G, glucuronic acid; A, *N*-trifluoroacetyl glucosamine; CH₃*, Residual *N*-acetyl;

Supplementary Figure 2



Supp. Fig. 2. ¹H NMR spectra of AGAIA (**bottom**) and AGAIA-CF3 (**top**) at 30 °C in D₂O. A, Glucosamine; A*, N-Sulfo,3,6-O-Sulfated Glucosamine; NR, reducing end, I2S, 2-O-Sulfated Iduronic Acida; R. end, Reducing end; G1, Glucuronic Acid. Signals are labelled according to reference J. Phys. Chem. B, 2015, 119 (38), pp 12397–12409.