Protein–GAG interactions: new surface-based techniques, spectroscopies and nanotechnology probes

E.A. Yates*, C.J. Terry*, C. Rees*, T.R. Rudd*†, L. Duchesne*, M.A. Skidmore*, R. Lévy*†, N.T.K. Thanh*†, R.J. Nichols†, D.T. Clarke‡ and D.G. Fernig*[1]

*Centre for Nanoscale Science, School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, U.K.,, †Centre for Nanoscale Science, Department of Chemistry, University of Liverpool, Liverpool L69 7ZB, U.K., and ‡CCLRC, Daresbury Laboratory, Warrington, Cheshire WA4 4AD, U.K.

Abstract

New approaches, rooted in the physical sciences, have been developed to gain a more fundamental understanding of protein–GAG (glycosaminoglycan) interactions. DPI (dual polarization interferometry) is an optical technique, which measures real-time changes in the mass of molecules bound at a surface and the geometry of the bound molecules. QCM-D (quartz crystal microbalance-dissipation), an acoustic technique, measures the mass and the viscoelastic properties of adsorbates. The FTIR (Fourier-transform IR) amide bands I, II and III, resulting from the peptide bond, provide insight into protein secondary structure. Synchrotron radiation CD goes to much shorter wavelengths than laboratory CD, allowing access to chromophores that provide insights into the conformation of the GAG chain and of β-strand structures of proteins. To tackle the diversity of GAG structure, we are developing noble metal nanoparticle probes, which can be detected at the level of single particles and so enable single molecule biochemistry and analytical chemistry. These new approaches are enabling new insights into structure–function relationships in GAGs and together they will resolve many of the outstanding problems in this field.

Introduction

The structural richness of GAGs (glycosaminoglycans) arises from the diversity of their saccharide sequences, the length of the GAG chains and the multiplicity of conformations that, in turn, depend on the complex interplay between linkage, ring geometry and substitution pattern. The consequently large information space provided by GAGs is exploited by biology to co-ordinate intercellular communication [1,2], illustrated by the hundreds of proteins whose activities are regulated by interactions with GAGs. In addition, their predominance at the cell surface and in the pericellular matrix [1] means that GAGs physically dominate this environment, in which intercellular communication takes place. Thus a major challenge for glycomics is to decipher structural details of GAG–protein interactions and, ultimately, the structure of entire GAG chains. An understanding of structure must encompass knowledge of the saccharide sequences involved in protein binding, the conformation of these sequences and that of the entire GAG chain, as well as the effects of GAG binding on protein structure, all in an environment as close as possible to that found *in vivo*. Such a challenge requires new approaches. Our strategy has been to assemble a set of tools, which originate in the physical sciences, to gain a more fundamental understanding of protein–GAG interactions. There are three strands to this toolkit: (i) new surface-based techniques, which enable the quantification of the three-dimensional organization of GAG chains in solution and of GAG–protein complexes; (ii) spectroscopic techniques, which report polysaccharide conformation and, when in a complex, that of the protein partner; and (iii) nano-biotechnology probes of a size equivalent to the smallest proteins, allowing the quantitative detection of single labelled GAG chains, oligosaccharides and proteins as well as providing a quantitative real-time readout on the assembly pathways and stoichiometry of GAG–protein complexes.

Novel surface techniques to analyse GAG chain structure and function

Two surface techniques, one optical, the other acoustic, provide information on the structures of GAGs and GAG–protein complexes that were hitherto inaccessible.

DPI (dual polarization interferometry)

DPI is an optical technique, which measures real-time changes in the mass of molecules bound at a surface and the geometry of the bound molecules. DPI measures change in phase for both TE (transverse electric) and TM (transverse

Key words: dual polarization interferometry, fibroblast growth factor, nanoparticle, protein–glycosaminoglycan interaction, quartz crystal microbalance-dissipation, synchrotron radiation CD spectroscopy.

Abbreviations used: DPI, dual polarization interferometry; FGF, fibroblast growth factor; FTIR, Fourier-transform IR; GAG, glycosaminoglycan; HGF/SF, hepatocyte growth factor/scatter factor; HS, heparan sulphate; QCM-D, quartz crystal microbalance-dissipation; SRCD, synchrotron radiation CD.

[1]**To whom correspondence should be addressed, at School of Biological Sciences, Biosciences Building, Crown Street, University of Liverpool, Liverpool L69 7ZB, U.K. (email dgfernig@liv.ac.uk).**

©2006 Biochemical Society

magnetic) polarizations of light. For each individual polarization of light, these changes in phase can be resolved, using Maxwell's equations, to an infinite number of thickness and refractive index values; however, only one unique value for the thickness and for the refractive index of a layer of molecules is true for both phases of light [3,4]. This means that instead of relative changes, which are measured in first generation optical biosensors based on surface plasmon resonance or evanescent waves [5], in DPI absolute measurements are made. Thus DPI is an extremely powerful technique, which allows a layer of molecules adsorbed to the surface of a sensor chip to be fully characterized in terms of mass, thickness and refractive index in real time, as the layer is formed. Moreover, glycans, whose refractive index differs from that of proteins and which depends on the level of sulphation, can be quantified directly in DPI. A simple application of the technique is illustrated by the immobilization of streptavidin on a biotinylated sensor surface, followed by the capture of reducing end biotinylated heparin oligosaccharides. DPI shows that the oligosaccharides do not extend into the bulk solution, as expected [6,7], but instead lie down in a layer approx. 4 nm thick, of which just 2 nm protrudes above the streptavidin layer. Given that membrane mimetics can be formed and characterized *in situ* [8,9], DPI has the potential to determine the arrangement of the GAG chains of proteoglycans in solution, the effects of ligands on these chains and the geometry of complexes of GAGs and proteins.

QCM-D (quartz crystal microbalance-dissipation)

QCM-D, an acoustic technique, measures the mass and the viscoelastic properties of adsorbates. In QCM-D [10], changes in the frequency of oscillation of a quartz crystal are caused by changes in mass adsorbing to the sensor surface [11]. As well as measuring changes in mass from an interaction, QCM-D also measures the dissipation of the bound molecules. Dissipation is a measure of the change in viscoelastic properties of the surface, which can be reduced in simple form to a change in flexibility [10,12]. Usually gold crystals are used in QCM-D. These are readily functionalized with thiol-containing molecules [13]; for example, 11-mercaptoundecanoic acid readily forms a self-assembled monolayer on the gold crystal surface [14]. The carboxylic acid groups are then functionalized with hydrazine to allow coupling of oligosaccharides via their reducing ends. Alternatively, a thiol can be coupled with the carboxylic acid groups to allow the coupling of Hg^+-oligosaccharides [15]. As with the DPI, membrane mimetics can also be formed [16,17], to enable proteoglycans with lipid anchors, e.g. glypicans, to be immobilized on the sensor surface in an environment that approximates that of the cell surface. These approaches then provide a platform to investigate the changes in mass and molecular flexibility that occur when proteins are bound to oligosaccharides or to GAG chains of proteoglycans. QCM-D thus provides a unique means to explore the flexibility of oligosaccharides, of GAG chains, of entire proteoglycans and of their complexes with proteins. It is therefore an excellent complement to DPI, which provides measurements of the orientation of molecules on the sensor surface.

Examples of the application of QCM-D include the measurement of binding of a series of growth factors, FGF-1 (fibroblast growth factor 1), FGF-2, FGF-7 and HGF/SF (hepatocyte growth factor/scatter factor) to heparin-derived oligosaccharides. The results of the present study show that the different protein–oligosaccharide complexes possess different flexibilities. For example, among these growth factors, the complex of FGF-1 with an octasaccharide is 1.5-fold more flexible per unit mass than the corresponding complex formed with HGF/SF.

Spectroscopic approaches

Spectroscopic methods, particularly CD and FTIR (Fourier-transform infrared) spectroscopy, are important tools for the characterization and measurement of secondary structural changes in protein–ligand interactions in solution [18]. Previously, these spectroscopies have been successfully used with relatively small ligands, such as drug molecules and hormones, which do not interfere significantly with the protein spectrum. We have recently embarked on an ambitious programme of investigations employing state-of-the-art FTIR and SRCD (synchrotron radiation CD) spectroscopy (T.R. Rudd, M.A. Skidmore, L. Duchesne, M. Guerrini, A. Naggi, R.J. Nichols, J.E. Turnbull, D.T. Clarke, D.G. Fernig and E.A. Yates, unpublished work), involving the study of protein interactions with a library of analogues [19] of HS (heparan sulphate).

CD

CD arises when a chromophore in an asymmetric environment absorbs right and left circularly polarized light, through the excitation of electrons in molecular orbitals, to a different extent. CD can be used to quantify protein secondary structure; α-helices, β-sheets, turns and unstructured regions all exhibit characteristic spectra [20,21] and changes in tertiary structure can also be detected through chromophores in aromatic amino acid side chains. Several well-documented algorithms have been compiled [22–24], which allow quantification of protein secondary structure from CD spectra.

A recent technological advance has been the introduction of SRCD, which is many orders of magnitude more sensitive than conventional CD instrumentation. Facilities such as the CD12 beamline at Daresbury Laboratory (U.K.) have rejuvenated the subject for this reason, but also because lower wavelengths (down to 168 nm in practice) than were conventionally available (~190 nm) are accessible. These lower wavelengths are particularly sensitive to changes in β-strand secondary structure. Importantly, SRCD has also allowed the acquisition of spectra of heparin, HS and related sugars down to 170 nm. Our work in progress is the first systematic CD study of this class of molecules to be undertaken in solution (T.R. Rudd, M.A. Skidmore, L. Duchesne, M. Guerrini, A. Naggi, R.J. Nichols, J.E. Turnbull, D.T. Clarke, D.G. Fernig and E.A. Yates, unpublished work). Signals arise

©2006 Biochemical Society

principally from two chromophores; the amide bonds present in N-acetyl groups of glucosamine residues and the carboxylate groups of uronic acids. The technique has also highlighted a number of distinct modes of cation binding to heparin. In some cases, the contribution of HS chromophores to spectra of protein–HS complexes is negligible but, if present to a significant extent, HS can interfere in their secondary structure analysis. One approach is to record the SRCD spectrum of the HS ligand under identical conditions with that of the protein–HS ligand complex and then subtract the former from the latter. This makes a number of assumptions. The first is that there is no significant change in the SRCD spectrum arising from the interaction of amino acid side chains with the HS ligand. The second is that there is no change in the HS ligand that alters SRCD spectral features. The first of these is probably untestable but we have found that the second could occur, inasmuch as the SRCD spectra of heparin derivatives, which are known through other spectroscopic techniques such as NMR [19,25] to possess modified uronic acid geometries, also exhibit altered SRCD spectra (T.R. Rudd, M.A. Skidmore, L. Duchesne, M. Guerrini, A. Naggi, R.J. Nichols, J.E. Turnbull, D.T. Clarke, D.G. Fernig and E.A. Yates, unpublished work).

FTIR

FTIR spectroscopy is a complementary technique [26] arising from the absorption of IR radiation through resonance of non-centrosymmetric (IR active) modes of vibration and is a useful tool for quantifying secondary structure in proteins. The position of the amide I band (between 1600 and 1700 cm^{-1} arising from C=O stretching) of the peptide backbone is sensitive to the short-range order imposed by distinct hydrogen-bonding arrangements in protein secondary structure and can be quantified by deconvolution techniques [27,28]. In the presence of HS, this region can coincide with signals from amide bands originating in the N-acetyl groups of glucosamine residues, but these can probably be safely subtracted from the spectrum of the complex because they are likely to be relatively insensitive to conformational changes in the saccharide. Deuteration of the protein and protein–HS complex following exchange with $^{2}H_2O$ can aid the process of assignment, e.g. amide II′ bands, due to the difference between N–H and N–D bending move from approx. 1546 to 1430/55 cm^{-1}. The bands of interest are near the middle of the spectral range, which is relatively uncluttered, but attempts to quantify the different secondary structural types are hampered by uncertainty over the extent of deuteration. Amide III bands (1350–1200 cm^{-1}) [29], which arise from several modes of vibration in the amide chromophore of the protein backbone, are also sensitive to secondary structural type; α-helix (1328–1289 cm^{-1}), β-sheet (1255–1224 cm^{-1}) and random coil (1288–1256 cm^{-1}). These can also be quantified but, in the presence of HS/heparin, are less useful because they coincide with strong sulphate stretches (centred between 1230–1240 and approx. 1360 cm^{-1}). There is also some overlap between the positions of some spectral bands arising from particular secondary structural features, particularly in the case of amide I and II′ bands. Subtraction of HS FTIR spectra (particularly in the amide I region) from that of the complex is probably currently the best approach, if analysis of the protein secondary structure is required in the presence of HS ligands, although the limitations and assumptions inherent in such an approach should be borne in mind.

Metal nanoparticles: single molecule probes for GAG structure and function

To tackle the diversity of GAG structure, we are developing noble metal nanoparticle probes, which can be detected at the level of single particles and so enable single molecule biochemistry and analytical chemistry.

Colloidal gold particles have a long-standing tradition as high-density labelling agents for electron microscopy of biological samples [30]. With the recent advent of nanotechnology, there has been a burgeoning renaissance of studies of colloidal gold and other metals (nanoparticles), which has been driven, in part, by the optical properties of gold and silver nanoparticles. Surface plasmons in metal nanoparticles (collective oscillations of electrons confined within the particle) have a characteristic resonance frequency, depending on particle size. Nanoparticles absorb, emit and scatter light in a manner characteristic of their size; bringing two or more particles close together (~10 nm) results in dipole coupling and a substantial red shift in plasmon resonance frequency. These unusual optical properties, which are not subject to quenching/photobleaching (fluorophores) or blinking (Q-dots), allow the detection of single particles and of interactions of particles brought about by, e.g., a linked biomolecular binding event, and have led to completely new perspectives of developing bio-analytical and imaging techniques [31]. Indeed, the development of photothermal imaging [32,33] and in particular the photothermal heterodyne microscope [34,35] makes nanoparticles one of the choice probes for single molecule biochemistry.

To exploit the optical properties of metal nanoparticles, protein-like nanoparticles have been developed in which short peptides of specific sequence self-assemble on the nanoparticle surface to protect it from aggregation and non-specific binding [36]. The power of this class of peptide ligand shells is that stabilization and functionalization of the nanoparticle are independent (different peptides), but achieved in the same preparative step [36,37]. Crucially for biological applications, the number of functions on the surface of the nanoparticles can be controlled, such that it is experimentally easy, using standard protein affinity chromatography techniques to produce nanoparticles with a defined number of functions [38]. Generally, for molecular labelling, univalently functionalized nanoparticles are required.

For labelling GAG-derived oligosaccharides, oligosaccharides produced by the action of lyases and activated at their non-reducing ends by Hg^{+} have been used [15]. The Hg^{+}-oligosaccharides can then be reacted with nanoparticles carrying either a thiol or a thioether, in practice a cysteine

©2006 Biochemical Society

or methionine residue respectively. The advantage of using the non-reducing end for coupling is that the reactions are far more efficient (near stoichiometric) compared with those with the reducing end [39].

Perspectives

Although many of these new approaches are still in development, we believe that together they form a high powered, complementary set of tools that will enable radical new insights into structure–function relationships in GAGs and together they will resolve many of the outstanding problems in this field.

We thank the Biotechnology and Biological Sciences Research Council, the Cancer and Polio Research Fund, the Council for the Central Laboratory of the Research Councils, the Human Frontiers Research Programme, the Medical Research Council (U.K.) and the North West Cancer Research Fund for financial support.

References

1 Delehedde, M., Lyon, M., Sergeant, N., Rahmoune, H. and Fernig, D.G. (2001) J. Mammary Gland Biol. Neoplasia **6**, 253–273
2 Turnbull, J., Powell, A. and Guimond, S. (2001) Trends Cell Biol. **11**, 75–82
3 Cross, G.H., Reeves, A.A., Brand, S., Popplewell, J.F., Peel, L.L., Swann, M.J. and Freeman, N.J. (2003) Biosens. Bioelectron. **19**, 383–390
4 Swann, M.J., Peel, L.L., Carrington, S. and Freeman, N.J. (2004) Anal. Biochem. **329**, 190–198
5 Fernig, D.G. (2001) in Proteoglycan Protocols (Iozzo, R.V., ed.), pp. 505–518, Humana Press, Totowa, NJ
6 Delehedde, M., Lyon, M., Gallagher, J.T., Rudland, P.S. and Fernig, D.G. (2002) Biochem. J. **366**, 235–244
7 Delehedde, M., Lyon, M., Vidyasagar, R., McDonnell, T.J. and Fernig, D.G. (2002) J. Biol. Chem. **277**, 12456–12462
8 Popplewell, J., Freeman, N., Carrington, S., Ronan, G., McDonnell, C. and Ford, R.C. (2005) Biochem. Soc. Trans. **33**, 931–933
9 Terry, C.J., Popplewell, J.F., Swann, M.J., Freeman, N.J. and Fernig, D.G. (2005) Biosens. Bioelectron., doi:10.1016/j.bios.2006.01.021
10 Rodahl, M., Hook, F., Fredriksson, C., Keller, C.A., Krozer, A., Brzezinski, P., Voinova, M. and Kasemo, B. (1997) Faraday Discuss. **107**, 229–246
11 Sauerbrey, G. (1959) Z. Phys. **155**, 206–222
12 Hook, F., Kasemo, B., Nylander, T., Fant, C., Sott, K. and Elwing, H. (2001) Anal. Chem. **73**, 5796–5804
13 Ulman, A. (1996) Chem. Rev. **96**, 1533–1554
14 Chidsey, C.E.D. and Loiacono, D.N. (1990) Langmuir **6**, 682–691
15 Skidmore, M.A., Patey, S.J., Thanh, N.T.K., Fernig, D.G., Turnbull, J.E. and Yates, E.A. (2004) Chem. Commun. **23**, 2700–2701
16 Keller, C.A. and Kasemo, B. (1998) Biophys. J. **75**, 1397–1402
17 Hook, F., Ray, A., Norden, B. and Kasemo, B. (2001) Langmuir **17**, 8305–8312
18 Middaugh, C.R., Mach, H., Burke, C.J., Volkin, D.B., Dabora, J.H., Tsai, P.K., Bruner, M.W., Ryan, J.A. and Marfia, K.E. (1992) Biochemistry **31**, 9016–9024
19 Yates, E.A., Santini, F., Guerrini, M., Naggi, A., Torri, G. and Casu, B. (1996) Carbohydr. Res. **294**, 15–27
20 Jones, G.R. and Clarke, D.T. (2004) Faraday Discuss. **126**, 223–236
21 Wallace, B.A. (2000) Nat. Struct. Biol. **7**, 708–709
22 Greenfield, N.J. (1996) Anal. Biochem. **235**, 1–10
23 Greenfield, N.J. (1999) TRAC Trends Anal. Chem. **18**, 236–244
24 Whitmore, L. and Wallace, B.A. (2004) Nucleic Acids Res. **32**, W668–W673
25 Yates, E.A., Santini, F., De Cristofano, B., Payre, N., Cosentino, C., Guerrini, M., Naggi, A., Torri, G. and Hricovini, M. (2000) Carbohydr. Res. **329**, 239–247
26 Oberg, K.A., Ruysschaert, J.M. and Goormaghtigh, E. (2004) Eur. J. Biochem. **271**, 2937–2948
27 Manning, M.C. (2005) Exp. Rev. Proteomics **2**, 731–743
28 Surewicz, W.K., Mantsch, H.H. and Chapman, D. (1993) Biochemistry **32**, 389–394
29 Cai, S.W. and Singh, B.R. (1999) Biophys. Chem. **80**, 7–20
30 Hayat, M.A. (1989) Colloidal Gold, Academic Press, San Diego
31 Doty, R.C., Fernig, D.G. and Lévy, R. (2004) Cell. Mol. Life Sci. **61**, 1843–1849
32 Cognet, L., Tardin, C., Boyer, D., Choquet, D., Tamarat, P. and Lounis, B. (2003) Proc. Natl. Acad. Sci. U.S.A. **100**, 11350–11355
33 Boyer, D., Tamarat, P., Maali, A., Lounis, B. and Orrit, M. (2002) Science **297**, 1160–1163
34 Berciaud, S., Cognet, L., Blab, G.A. and Lounis, B. (2004) Phys. Rev. Lett. **93**, 257402
35 Berciaud, S., Cognet, L. and Lounis, B. (2005) Nano Lett. **5**, 2160–2163
36 Lévy, R., Thanh, N.T.K., Doty, R.C., Hussain, I., Nichols, R.J., Schiffrin, D.J., Brust, M. and Fernig, D.G. (2004) J. Am. Chem. Soc. **126**, 10076–10084
37 Wang, Z., Lévy, R., Fernig, D.G. and Brust, M. (2005) Bioconjugate Chem. **16**, 497–500
38 Lévy, R., Wang, Z., Duchesne, L., Doty, R.C., Cooper, A.I., Brust, M. and Fernig, D.G. (2006) Chembiochem **7**, 592–594
39 Yates, E.A., Jones, M.O., Clarke, C.E., Powell, A.K., Johnson, S.R., Porch, A., Edwards, P.P. and Turnbull, J.E. (2003) J. Mater. Chem. **13**, 2061–2063

Received 20 December 2005

©2006 Biochemical Society