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Real-time monitoring of adherence of *Streptococcus anginosus* group bacteria to extracellular matrix decorin and biglycan proteoglycans in biofilm formation

Janine Landrygan-Bakri, Melanie J. Wilson, David W. Williams, Michael A.O. Lewis, Rachel J. Waddington*

Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff CF14 4XY, UK

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Abstract

Members of the *Streptococcus anginosus* group (SAGs) are significant pathogens. However, their pathogenic mechanisms are incompletely understood. This study investigates the adherence of SAGs to the matrix proteoglycans decorin and biglycan of soft gingival and alveolar bone. Recombinant chondroitin 4-sulphate(C4S)-conjugated decorin and biglycan were synthesised using mammalian expression systems. C4S-conjugated decorin/biglycan and dermatan sulphate (DS) decorin/biglycan were isolated from ovine alveolar bone and gingival connective tissue, respectively. Using surface plasmon resonance, adherence of the SAGs *S. anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* to immobilised proteoglycan was assessed as a function of real-time biofilm formation. All isolates adhered to gingival proteoglycan, 59% percent of isolates adhered to alveolar proteoglycans, 70% to recombinant decorin and 76% to recombinant biglycan. Higher adherence was generally noted for *S. constellatus* and *S. intermedius* isolates. No differences in adherence were noted between commensal and pathogenic strains to decorin or biglycan. DS demonstrated greater adherence compared to C4S. Removal of the GAG chain with chondroitinase ABC resulted in no or minimal adherence for all isolates. These results suggest that SAGs bind to the extracellular matrix proteoglycans decorin and biglycan, with interaction mediated by the conjugated glycosaminoglycan chain.

Keywords: *Streptococcus anginosus* group; Decorin; Biglycan; Dermatan sulphate; C4S; Surface plasmon resonance

1. Introduction

The *Streptococcus anginosus* group (SAG) comprises microaerophilic bacteria which are generally regarded as members of the commensal flora of the body (Gossling, 1988; Whiley et al., 1992). However, SAG members are frequently isolated from a range of clinical sites, including liver and brain abscesses, infective endocarditis and infections of the gastrointestinal and genitourinary tract. Whilst present in low numbers in dental plaque, SAGs are frequently isolated from polymicrobial infections; they are predominant components of a number of orofacial infections, including dento-alveolar and periodontal abscesses, and are frequently associated with failed root canal treatment (Jacobs et al., 2003; Ledezma-Rasillo et al., 2010; Lewis et al., 1986; Schuman and Turner, 1999; Siqueira et al., 2002; Okayama et al., 2005; Whiley et al., 1992). It has been proposed that SAGs are present early in the pathogenic process and may actually initiate infection, thereafter preparing the environment for subsequent colonisation by anaerobic species (Aderhold et al., 1981; Gossling, 1988; Nagashima et al., 1999; Shinzato and Saito, 1994). As microaerophilic bacteria, SAGs can

* Corresponding author. Tel.: +44 (0) 29 2074 2609.
E-mail addresses: jan_bakri@yahoo.co.uk (J. Landrygan-Bakri), wilsonmj@cardiff.ac.uk (M.J. Wilson), Williamsdd@cardiff.ac.uk (D.W. Williams), lewismao@cardiff.ac.uk (M.A.O. Lewis), waddingtonrj@cardiff.ac.uk (R.J. Waddington).

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proliferate in regions of low oxygen tension, further reducing both the oxygen concentration and redox potential to favour the growth of strict anaerobes. They have a marked propensity for haematogenous spread and thus infection involving SAGs is often complicated by seeding of the infective process elsewhere in the body (Gossling, 1988). Despite being almost universally susceptible to penicillin, infections involving SAGs are often difficult to treat (Green et al., 2001; Han and Kerschner, 2001; Schuman and Turner, 1999). Although a number of potential virulence factors have been investigated, including platelet aggregation, fibrinogen and fibrin binding, haemolytic and hydrolytic activity, the pathogenesis of SAGs remains poorly understood (Jacobs et al., 2000; Kitada et al., 1997; Shain et al., 1996; Willcox, 1995).

The extracellular matrix (ECM) of connective tissue provides a prime site for the adherence or attachment of bacteria in the establishment of infection. The principle elements may be considered as a collagen fibrous network providing structural support, embedded in and interacting with a non-collagenous matrix consisting of proteoglycans (PGs) and various glycoproteins. Across the breadth of connective tissues, perhaps one of the most prominent PG families is that of the small-leucine rich proteoglycans (SLRPs) decorin and biglycan. As for all SLRPs, decorin and biglycan possess a common protein core domain structure, dominated by a central domain consisting of 6–10 leucine-rich repeat sequences flanked by an N-terminal and C-terminal region with a conserved cysteine pattern (Iozzo, 1999). Close to the N-terminal, decorin and biglycan are conjugated to one and two glycosaminoglycan (GAG) chains respectively. These GAG chains represent long polymer disaccharide repeat sequences of an uronic acid and an N-acetyl hexosamine, with molecular weight 20–40 kDa (Iozzo, 1999). Within mineralised matrices of bone and dentine, these GAG chains are predominantly characterised as the glucuronic acid-containing chondroitin sulphate (CS), whilst in soft connective tissues such as skin, ligament and tendon, the iduronic acid-containing dermatan sulphate (DS) predominates (Waddington and Embery, 1991). In addition, PGs are also present on the cell surface, with the most prominent members belonging to the syndecan family. These PGs are identified in multiple forms on the majority of cell and tissue types, although their expression is selectively regulated in a cell-, tissue- and development-dependent manner (Kim et al., 1994). Structurally, these molecules consist of a core protein which crosses the cell membrane, and the extracellular domain carries several heparin sulphate and/or chondroitin sulphate glycosaminoglycan chains.

When considering ECM/microbial interactions in the establishment of infection involving streptococcal species in general, much research in the literature has been restricted to establishing binding of bacteria to heparin sulphate and heparin (Rostand and Esko, 1997), with interactions facilitated via the microbial surface cell recognition adhesion matrix molecules (MSCRAMMs) present on the bacterial surface (Almeida et al., 1999a,b; Almeida et al., 2003; Egesten et al., 2011; Frick et al., 2003; Wang et al., 1993). There is evidence that the binding to these cell surface receptors may result in host cell responses, including intracellular signalling, influencing host cell survival, promotion of antimicrobial peptide synthesis and initiation of the inflammatory response (Baron et al., 2009; Hoepelman and Tuomanen, 1992; Jockusch et al., 1995; Wang et al., 2011), and thus they may be significant in the establishment of infection. Although still in their infancy, these studies have led to suggested options for therapeutic strategies related to the elucidation of microbe/PG interactions (Baron et al., 2009; Hoepelman and Tuomanen, 1992; Jockusch et al., 1995). Interactions with GAGs associated with decorin and biglycan, CS and DS, have been demonstrated for species *Streptococcus pyogenes* (Frick et al., 2003), *Streptococcus uberis* (Almeida et al., 1999a,b; Almeida et al., 2003) and *Streptococcus pneumonia* (Tonnaer et al., 2006). However, no studies have investigated binding of *Streptococcus* species to the protein cores of these PGs. Furthermore, no studies have investigated the interaction of these PGs with SAGs.

Against this background, the aim of this study was to explore the binding of various SAG of bacteria with the PGs decorin and biglycan. Specifically, this study will assess the attraction and binding strength of bacteria for PGs in real-time using surface plasmon resonance (SPR), thus assessing early events associated with bacterial biofilm formation and colonization. This study will investigate SAG isolates recovered from healthy body sites and clinical infections. These findings will be compared and interpreted in terms of the differential binding of the individual strains with the CS- and DS-conjugated forms of decorin and biglycan, present in mineralised and soft connective tissue.

2. Materials and methods

2.1. Recombinant proteoglycans

cDNAs for mouse decorin (mDCN-5) or biglycan (clone 3) cDNAs were subcloned in the mammalian expression vector pcDNA3.1 myc/his (Invitrogen) followed by liposomal transfection into HeLa cells as previously described by Sugars et al. (2002). Transfected cells were cultured in Eagles Minimum Essential Medium (EMEM) with Earles Balanced Salt Solution (EBSS), supplemented with 2 mM glutamine, 1% antibiotic/antimycotic solution and 2% foetal bovine serum (FBS) at 37 °C, 5% CO_2. Recombinant decorin or biglycan was purified from the retained media using 1 ml HiTrap nickel affinity chelating columns prepared as per manufacturing instructions (GE Healthcare). His-tag recombinant proteins were eluted with 0.02 M sodium phosphate, 0.5 M sodium chloride and 500 mM imidazole, pH 7.4, dialysed exhaustively against double-distilled water (4 days containing the protease inhibitors 5 mM benzamidine-HCl, 1 mM iodoacetic acid, 5 mM *N*-ethylmaleimide (Sigma Aldrich) and 1 day double-distilled water only) and recovered by lyophilisation.

Recombinant proteoglycans were further purified by anion exchange using a 1 ml Resource Q column (GE Healthcare) in

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order to remove co-eluting α_2HS-glycoprotein (Sugars et al., 2002). Recombinant proteoglycans were applied to the column in 7 M urea, 0.05 M tris–HCl buffer, pH 6.8 (1 mg/ml) and eluted using an expanded 0–1 M sodium chloride gradient. Pooled fractions were obtained according to peaks within the 280 nm profile, exhaustively dialysed against double-distilled water (with protease inhibitors as above), lyophilised and re-dissolved in double-distilled water to 20 mg/ml, which was adjusted following determination of protein concentration using the bicinchoninic acid (BCA) assay kit (Pierce Endogen Ltd., Cheshire, UK). 1 µl of each of the pooled fraction samples was applied to a nitrocellulose membrane (Hybond™ C, Amersham Pharmacia Biotech) which was then blocked in 10% non-fat milk (Marvel™), in TBS (10 mM tris–HCl, 0.15 M sodium chloride, pH 7.4) for 30 min. Proteoglycan-rich fractions were identified by incubation with mouse monoclonal anti-chondroitin sulphate, CS-56 (Sigma Aldrich; 1:200 dilution in 3% non-fat milk in TBS) for 1 h. Subsequent to washing with TBS, membranes were incubated with goat anti-mouse IgM conjugated to alkaline phosphatase (Sigma Aldrich; 1:10,000 dilution) for 1 h, washed with TBS and immunoreactivity was visualised using NBT/BCIP chromogenic substrate (Promega).

2.2. Isolation of proteoglycans from gingiva and alveolar bone

Gingiva was dissected 1–2 mm below the gingival-tooth junction around the upper and lower dentition of 9-month-old Welsh Mule breed sheep (within 1–2 h of slaughter at the local abattoir). Buccal plates were removed from both maxillae and mandibles using a dental drill, molar and premolar teeth were extracted and bone from the immediate areas around the sockets was removed using a surgical chisel. Proteoglycans were isolated from alveolar bone according to the method of Waddington and Embery (1991). Briefly, soft adherent tissue was removed from the bone pieces with collagenase dispase, dehydrated in ethanol washes, followed by diethyl ether to remove lipids and then left at room temperature for the residual solvent to evaporate. Bone was powdered at −20 °C, demineralised with 10% EDTA (trisodium salt) solution, pH 7.45 and non-collagenous material was extracted using 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.9 and containing above named protease inhibitors. Solubilised non-collagenous proteins were recovered by centrifugation at 1800 g, 15 min, dialysed against double-distilled water and lyophilised. Gingival tissue was washed in phosphate-buffered saline (PBS) then sequentially washed twice in ethanol, followed by diethyl ether for 10 min and left at room temperature for the solvent to evaporate. Non-collagenous gingival tissue components were extracted into 4 M guanidinium chloride extraction buffer and recovered as for treatment of alveolar bone tissue samples.

Non-collagenous protein extracts from gingival or alveolar bone were dissolved in 7 M urea, 0.05 M sodium acetate buffer, pH 6.8, at a concentration of 10 mg/ml 1 ml samples were applied to a HiLoad 16/10 Q-Sepharose column (GE Healthcare), integrated into a fast performance anion exchange chromatography system (GE Healthcare) and eluted with 7 M urea, 0.05 M sodium acetate buffer, pH 6.8, 2 ml/min. Bound material was selectively eluted with a linear 0–1 M sodium chloride gradient over 240 ml. Eluting fractions were pooled according to the protein peaks identified in the 280 nm profile, dialysed against double-distilled water (as above) and recovered by lyophilisation. Samples were dissolved to 20 mg/ml in double-distilled water, with the concentration adjusted following determination of the concentration using the BCA protein assay (Pierce Endogen Ltd., Cheshire, UK), and proteoglycan-rich fractions were identified by immunoreactivity with anti-chondroitin sulphate antibody using the dot blot assay described above.

2.3. Chondroitinase ABC digestion

Samples of recombinant decorin, biglycan or proteoglycan from gingival or alveolar bone were mixed with an equal volume of 0.2 M tris–HCl buffer, 0.06 M sodium acetate, pH 8 containing 2 units/ml chondroitinase ABC (protease-free, Seikagku Corporation, Tokyo) and incubated at 37 °C for 1–2 h. Core proteins were separated from chondroitinase ABC and GAG fragments using gel filtration chromatography on a pre-packed Sephadex 75 HR 10/30 column (Amersham Pharmacia Biotech.), eluted with 4 M urea, 0.05 M Tris–HCl, pH 8, containing 0.35 M sodium acetate, at a flow rate of 1 ml/min 280 nm absorbance was monitored and protein fractions were pooled, dialysed against ddH_2O with protease inhibitors (as described above) and lyophilised. Decorin or biglycan core protein fractions were identified by immunoreactivity with the polyclonal antibody rabbit anti-mouse decorin (LF-113; raised against C-terminus of decorin) or rabbit anti-mouse biglycan (LF-106; raised against C-terminus of biglycan) (Fisher et al., 1995) using the above described dot-blot analysis.

2.4. SDS PAGE and western blot analysis

Samples of intact proteoglycans and their respective core proteins were mixed with an equal volume of 0.125 M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-β-mercapthoethanol and 0.004% bromophenol blue and examined by SDS-PAGE using the Phastsystem (GE Healthcare) as previously described by Waddington et al. (1993). SDS-6H molecular weight markers (Sigma Aldrich) were included on each gel. Following separation, gels were stained using the Silver Stain Kit (GE Healthcare). For western blot analysis, separated gels were electroblotted onto a nitrocellulose membrane (Hybond™ C, Amersham Pharmacia Biotech) with transfer buffer, 25 mM Tris HCl buffer in 20% methanol, containing 192 mM glycine, pH 8.3. Membranes were blocked with 10% non-fat milk (Marvel™), in TBS (10 mM tris–HCl, 0.15 M sodium chloride, pH 7.4) for 30 min and then incubated with primary polyclonal antibody rabbit anti-mouse decorin (LF-113) or rabbit anti-mouse biglycan (both diluted 1:200 in 3% milk, TBS) for 1 h. Immunoreactivity was

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visualised following incubation with goat anti-rabbit IgG conjugated to horse radish peroxidase (diluted 1:1000 in 3% milk, TBS) and detection using the ECL Plus western blotting detection reagent kit (GE Healthcare).

2.5. Identification of GAG constituents

Samples of intact proteoglycans were digested with an equal volume of 10 mg/ml type XIV protease (Sigma Aldrich) in 0.2 M tris–HCl and 10 mM calcium chloride pH 7.5 and incubated at 55 °C for 20 h. Samples were subsequently recovered by lyophilisation, reconstituted with ddH_2O to the equivalent of the original starting volume of the PG sample and examined by cellulose acetate electrophoresis as previously described by Waddington et al. (2004). GAGs were identified as judged by their electrophoretic mobility against the standards hyaluronan (HA), heparin sulphate (HS), dermatan sulphate (DS), chondroitin 4-sulphate (C4S) and chondroitin 6 sulphate (C6S) (Sigma Aldrich) separated on the same membrane.

2.6. Bacterial source and preparation

Isolates of SAG and sources are detailed in Table 1. The isolates had previously been characterised and assigned to *Streptococcus constellatus*, *S. anginosus* or *Streptococcus intermedius* using a phenotypic identification scheme (Bartie et al., 2000; Whiley et al., 1990). Bacteria were subcultured on Fastidious Anaerobic Agar (FAA) (Lab M™ International Diagnostic Group plc, Bury, UK), supplemented with 5% v/v defibrinated sheep blood (TCS Bioscience Ltd., Buckingham, UK) and purity was confirmed regularly. Bacterial cells were inoculated into 10 ml of brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) and subsequently incubated overnight in an anaerobic cabinet (10% v/v CO_2, 20% v/v H_2, 70% v/v N_2), at 37 °C (Don Whitley Scientific Ltd., Shipley, UK) to the stationary growth phase (determined from growth curves experiments). Bacterial cells were harvested by centrifugation at 3000 g for 10 min and washed twice in filtered, degassed HBS-EP buffer (0.01 M Hepes, 0.15 M NaCl, 0.003 mM EDTA, pH 7.4, containing 0.005% surfactant P20). The cells were sonicated (60 W, 10 s) to disrupt the streptococcal chains and re-suspended in HBS-EP buffer to an $OD_{(550\ nm)} = 0.5$ (approximately 1×10^8 cfu/ml, determined by serial dilutions, plated onto FAA plates and colony counts following overnight incubation).

Table 1
Identity and source of SAG isolates used in this study. GAG depolymerase activity indicated where detected.

Species	Reference	Clinical source	GAG depolymerase activity detectable
S. constellatus	350/96	Dento-alveolar abscess	
S. constellatus	F436	Lung abscess	
S. constellatus	322/95	Dento-alveolar abscess	Positive for chondroitinase and hyaluronidase activity
S. constellatus	48C	Tongue	
S. constellatus	34C	Tongue	Positive for chondroitinase and hyaluronidase activity
S. intermedius	HW13	Dento-alveolar abscess	
S. intermedius	127/95	Dento-alveolar abscess	Positive for chondroitinase and hyaluronidase activity
S. intermedius	447/96	Dento-alveolar abscess	
S. intermedius	11C	Tongue	
S. intermedius	30C	Tongue	Positive for chondroitinase and hyaluronidase activity
S. intermedius	84C	Plaque	
S. anginosus	43586/96	High vaginal swab	
S. anginosus	39/2/14A	Unknown	
S. anginosus	670/95	Dento-alveolar abscess	
S. anginosus	16C	Plaque	
S. anginosus	19C	Plaque	
S. anginosus	43C	Tongue	

2.7. Detection of GAG depolymerase activity

Depolymerisation of GAG was assessed using the method of (Tipler and Embery, 1985). Briefly, 4 ml BHI broth (Gibco) supplemented with either chondroitin-4-sulphate (Whale cartilage, type A, Sigma Aldrich) or hyaluronic acid (bovine umbilical cord, grade 1, Sigma Aldrich), final concentration 400 μg/ml, was inoculated with 1 ml of bacterial suspension (harvested at mid-log phase of growth) and incubated under anaerobic conditions for 7 days. After incubation, samples were centrifuged at 3000 g for 10 min, 20 μl of each supernatant was added to 4 ml of distilled water and 232 nm absorbance (due to unsaturated bond formation following the elimination reaction of HA and CS) recorded against non-inoculated broth (negative controls).

2.8. Surface plasmon resonance analysis

The interactions between mixed SLRP preparations from ovine tissues and recombinant decorin and biglycan with the SAG isolates were investigated by real-time biomolecular interaction analysis (BIA) using surface plasmon resonance (SPR) technology on a BIAcore® 3000 system (BIAcore, Uppsala, Sweden) using a C1 sensorchip (BIAcore). The C1 chip has a carboxymethylated surface with no dextran matrix and is useful when studying interactions where the analyte is large, such as bacteria. Recombinant proteoglycans or tissue-extracted proteoglycans (ligand) were dissolved in 10 mM phosphate buffer, pH 7.1 (150 μg/ml; Biacore), whilst deglycosylated protein cores were dissolved in 10 mM sodium acetate buffer (150 μg/ml; BIAcore). At a flow rate of 10 μl/min, the docked chip was first treated with BIA normalisation solution (70% w/w glycerol in water) (BIAcore) to create a standardised total reflection and then equilibrated with HBS-EP buffer. Proteoglycan ligands were immobilised on the sensor chip surface of flow cell 2 using amino coupling by first treating the surface (to produce active ester groups) by injecting 70 μl of 400 mM 1-ethyl-3-

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(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/70 µl 100 mM N-hydroxysuccinimide (NHS) solutions. After activation, 35 µl of 150 µg/ml of ligand in appropriate buffer was applied to the surface. The system was washed through with HBS-EP buffer to remove any loosely associated ligand. Finally, residual NHS esters were inactivated with 70 µl of 1 M ethanolamine hydrochloride, pH8.5. Flow cell 1 was used as an in-line reference for flow cell 2, where the blank surface was exposed to amine coupling in the absence of ligand. Thirty-five µl of fibronectin 10 mM sodium acetate buffer (150 µg/ml; Biacore) coupled to the surface served as a positive control. Bacterial cells within the HBS-EP buffer, pH 7.4 were injected over the various ligand-prepared surfaces at 20 µl/min for 125 s at 25 °C, followed by a 5 min dissociation phase (HBS-EP buffer only). The bacteria/ligand interaction produces an increase in the SPR signal, measured as a response unit (RU). Residual bound bacteria was washed off with 100 mM NaOH, pH 9.5 for 1 min and the chip surface re-equilibrated with HBS-EP buffer for 5 min between the application of bacterial test isolates. Each isolate was analysed in triplicate in a randomised order.

2.9. Statistical analysis

The RU data obtained was assessed by analysis of variance (ANOVA) using SPSS 12. The data was evaluated both in terms of differences between strains for a specific substrate and differences for substrates within a particular strain. Differences were considered significant at $p < 0.05$ and data expressed as the mean value of triplicate assays ± standard deviation.

3. Results

3.1. Characterisation of proteoglycan ligands

Recombinant proteoglycans were synthesised by transfected HeLa cells and purified from the culture media by nickel affinity chromatography. Previous studies demonstrated that recombinant proteoglycans copurify with α_2HS glycoprotein which is present within the serum of the culture media (Sugars et al., 2002). Recombinant decorin and biglycan were therefore further purified by anion exchange chromatography using a resource Q column. A substantial amount of α_2HS glycoprotein was observed to elute from the column prior to application of the sodium chloride gradient and recombinant decorin or biglycan eluted at 0.3–0.35 M sodium chloride. The elution profiles were consistent with those previously published as part of detailed descriptions for the purification protocols of these proteoglycans (Sugars et al., 2002). Silver-stained SDS-PAGE gels of the purified recombinant proteoglycan are shown in Fig. 1A. For recombinant biglycan, protein bands were prominent at approximately 98, 66 and 45 kDa, whilst for recombinant decorin, bands were prominent at 66 and 45 kDa. Digestion of either recombinant decorin or biglycan with chondroitinase ABC to remove the GAG chains yielded a single band at 45 kDa, consistent with the known molecular weight for the respective core proteins. Western blot analysis confirmed the immunoreactivity of the 45 kDa band with antibodies against decorin or biglycan, respectively (Fig. 1B). Both recombinant biglycan and decorin were identified as C4S-rich, as demonstrated by their immunoreactivity with monoclonal antibody CS56 (Fig. 1C). Previous

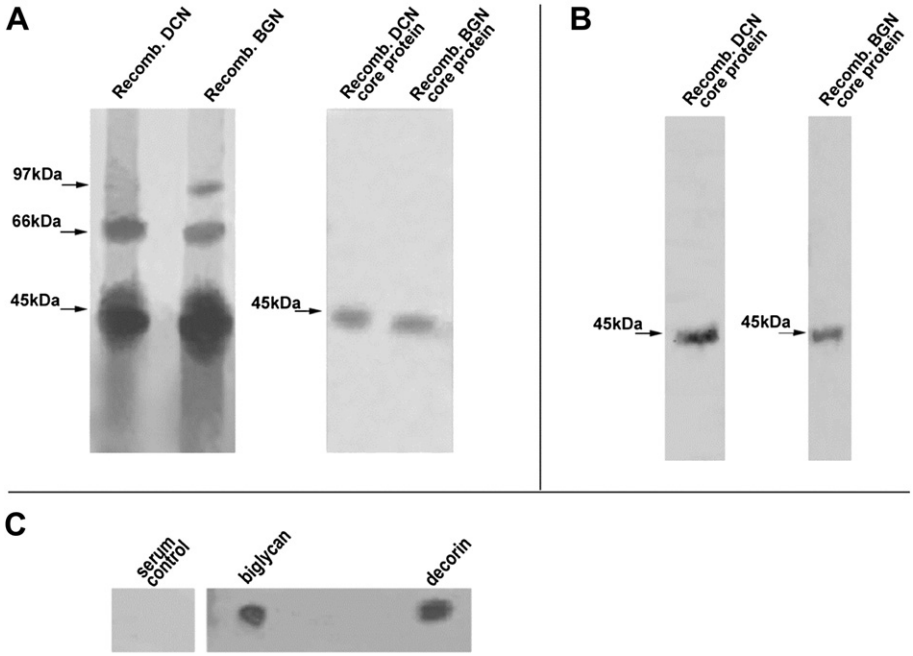

Fig. 1. Characterisation of recombinant proteoglycans, decorin and biglycan. Proteoglycans were synthesised within a mammalian expression system and purified by anion exchange chromatography. Core proteins relating to either decorin or biglycan were detected by SDS PAGE (A) or western blot analysis (B). The presence of chondroitin sulphate chains was confirmed by immunoreactivity to the monoclonal antibody CS56. DCN: decorin; BGN: biglycan.

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analysis had indicated that recombinant decorin and biglycan are synthesised with C4S chains, representing 76% of the total GAG content. In addition, chondroitin sulphate chains with reduced sulphation were also identified (approx 24% total GAG) (Sugars et al., 2002).

Non-collagenous proteins isolated from ovine gingiva or alveolar bone were fractionated using a two-stage anion exchange chromatography procedure. 280 nm absorbance profiles were similar to those previous reported for isolation of proteoglycans from connective tissues (Waddington et al., 1993, 2003) and have proven successful in purifying proteoglycans from other glycoprotein matrix components. For extracts from gingival and alveolar bone, a broad proteoglycan-rich fraction eluted from Q Sepharose with sodium chloride concentrations within the range of 0.3–0.4 M. These proteoglycan-rich fractions were further purified with Resource Q, eluting with 0.35 M sodium chloride. Silver-stained SDS PAGE analysis for alveolar bone proteoglycan indicated a protein band at approximately 45 kDa with strong staining for protein material also identified within the molecular weight range of 220–55 kDa, representative of proteoglycan substituted by a GAG chain of heterogeneous length (Fig. 2A). This was confirmed by the appearance of a single 45 kDa band after digestion of the GAG chains with chondroitinase ABC. Western blot analysis confirmed this 45 kDa band to be the core proteins of decorin and biglycan (Fig. 2B). Following removal of the protein core by protease digestion, the GAG chains were analysed by cellulose acetate. Electrophoretic mobility of the released GAGs compared to commercial GAG standards confirmed that decorin and biglycan isolated from alveolar bone were substituted by C4S. Analysis of gingival proteoglycans on silver-stained SDS PAGE gels identified two strong bands at approximately 45 kDa. In addition a sharp band was observed at approximately 50 kDa and protein staining materials were apparent at the top of the gel (molecular weight 220 kDa), which were lost following removal of the GAG with chondroitinase ABC (Fig. 2A). Western blot analysis again confirmed the 45 kDa band to be core proteins decorin and biglycan (Fig. 2B). Cellulose acetate electrophoresis of the GAG chains (core protein digested with protease) indicated that these proteoglycans were substituted by C4S and DS.

3.2. Interaction of SAG with proteoglycans and fibronectin

Fig. 3 shows typical sensograms obtained following injection of various SAG isolates over immobilised gingival proteoglycans (Fig. 3A) or fibronectin (Fig. 3B). For all ligands, sensograms recorded specific interactions of the SAG isolates with the proteoglycan ligand. No interaction events were reported for the flow of SAG isolates over the control cell containing no ligand, indicating that, in these conditions, the bacteria were unable to bind to the surface of the chip and did

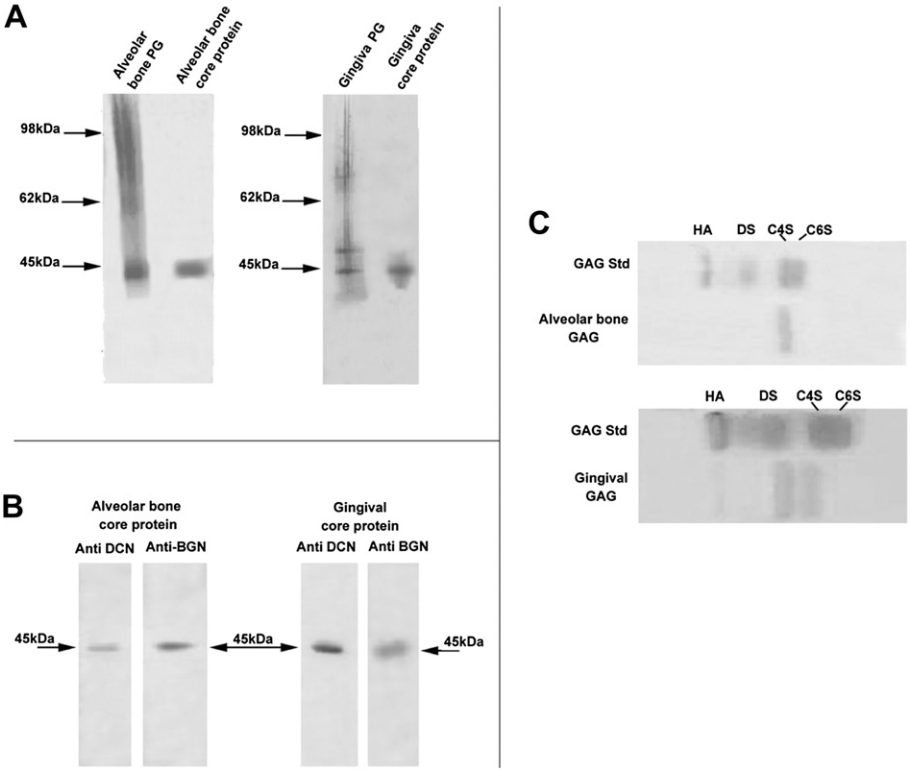

Fig. 2. Characterisation of the proteoglycans decorin and biglycan from alveolar bone and gingival connective tissues. Non-collagenous proteins were extracted from demineralised tissues with 4 M guanidinium chloride and proteoglycans were purified by anion exchange chromatography. Proteoglycan-rich fractions containing both decorin and biglycan were characterised by SDS PAGE (A) and western blot analysis. The GAG component conjugated to the proteoglycans was identified by cellulose acetate electrophoresis (C). DCN: decorin; BGN: biglycan.

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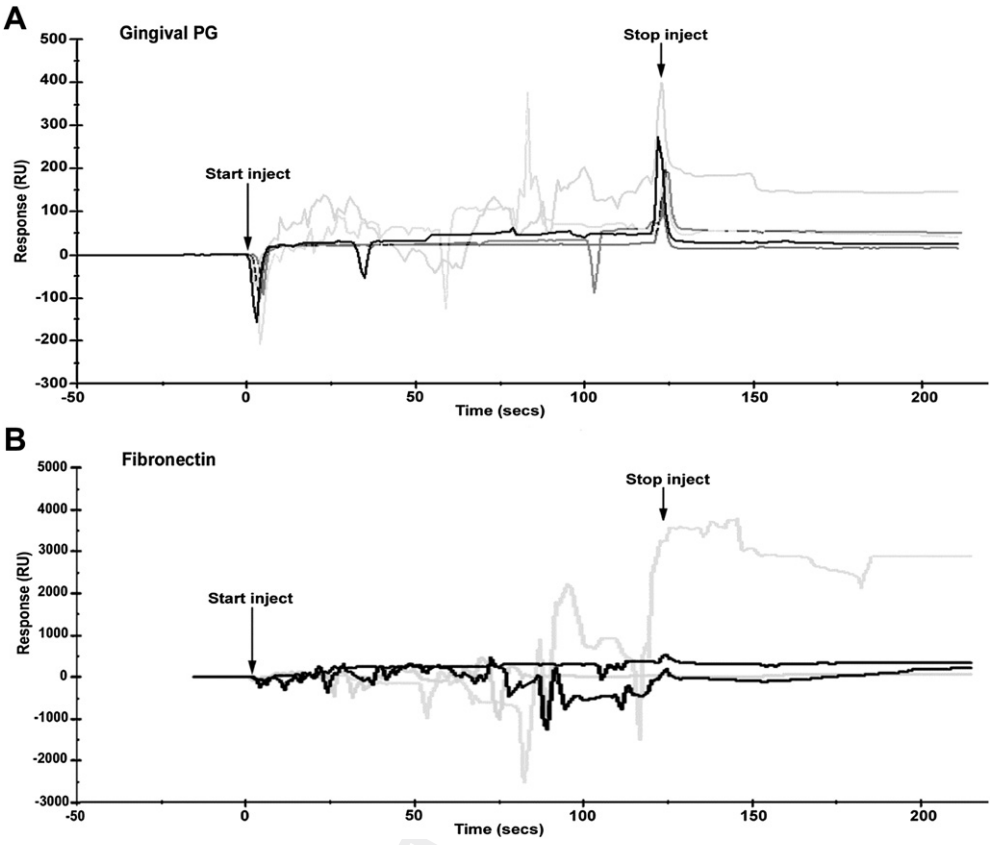

Fig. 3. Typical SPR sensograms obtained following injection of various SAG isolates (*S. constellatus*, F436, 322/95, 48C and *S. anginosus* 39/2/14A and 19C shown) over immobilised gingival proteoglycans (A) or fibronectin (B). Proteoglycan was chemically immobilised to a C1 sensorchip (BIAcore) and SAG isolates (1×10^8 cfu/ml) were injected over the immobilised proteoglycans and bacterial/ligand interaction measured as a response unit (RU). Fibronectin provided a positive control. Adherence was taken as the entire process of attachment and accumulation of bacteria in the formation of biofilm on the surface and measured as the difference in RU value from before injection of cells to the end of injection.

not form large aggregates which could be recorded by surface resonance technology. Sensograms for bacterial/ligand interactions produced fluctuating profiles representing a series of random association and dissociation events. This is likely to be due to the SAG isolate forming electrostatic interactions with the proteoglycan ligand, which are readily broken by the shear force generated by the eluting buffer flowing over the surface. This prevented calculation of affinity and dissociation constants. In this study, the term adherence was therefore taken as the entire process of attachment and accumulation of bacteria in formation of a biofilm on the surface, considering both initial cell-to-surface adherence and subsequent cell-to-cell adherence of bacterial cells (Eifukukoreeda et al., 1991). Adherence was observed as the difference in RU value from immediately before injection of cells (binding response at baseline) to the end of injection. Of note, the majority of strains that bound to the ligand surface to form a biolayer did not dissociate immediately from the surface following the end of injection, but required the application of 100 mM sodium hydroxide to remove bound bacteria and return the RU to baseline.

Mean RU values ± standard deviations from triplicate assays are presented in Table 2 and graphically in Fig. 4, illustrating trends in adherence patterns to the respective proteoglycan ligands. All strains demonstrated the ability to adhere to fibronectin, which was included as a positive control. For the *S. anginosus* commensal strain, 19C, 16C, 43C, *S. constellatus* commensal strain 34C, pathogenic strain F436 and *S. intermedius* pathogenic strains 127/95, HW13 and 447/95, adherence to fibronectin was significantly higher compared to adherence to the respective proteoglycan ligands ($p < 0.01$ for all comparisons). Gingival proteoglycans showed adherence for all SAG strains, with significantly greater adherence to gingival proteoglycans compared with other proteoglycans noted for *S. anginosus* 43C, 43586/96, 670/95, 39/2/14A, *S. constellatus*, 48C, F436, 322/95and *S. intermedius*, 84C447/95 ($p < 0.01$ for all comparisons). Adherence of *S. anginosus* 39/2/14A and *S. constellatus* 48C to gingival proteoglycans was significantly higher compared to adherence to fibronectin ($p < 0.05$ for both comparisons). 59% percent of strains, which included both commensal and pathogenic isolates, demonstrated some ability to adhere to alveolar proteoglycans, 70% of strains bound to intact recombinant decorin and 76% bound to recombinant biglycan. Almost all members of the *S. intermedius* group were able to adhere to all proteoglycan ligands examined, with all commensal strains showing strong adherence to recombinant decorin and biglycan and alveolar bone and gingival proteoglycans ($p < 0.01$ when compared

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Table 2
Mean response units (RUs) for strains of *S. anginosus*, *S. constellatus* and *S. intermedius* to recombinant and tissue-extracted proteoglycans, their respective core proteins and fibronectin. Means determined from three independent assays (±SD). Response units were determined as the increase in the SPR signal after 125 s of injection of bacterial isolates compared with the SPR signal at start of injection.

Strain	Recombinant decorin		Recombinant biglycan		Gingiva		Alveolar bone		Fibronectin
	Intact	Core protein	Intact	Core protein	Intact	Core protein	Intact	Core protein	
S. anginosus									
19C	NBD	NBD	NBD	NBD	41.7 ± 25.1	NBD	32.9 ± 20.2	NBD	289 ± 23.4
16C	NBD	NBD	NBD	NBD	95.8 ± 45.8	NBD	4.7 ± 1.4	NBD	2170.2 ± 245.3
43C	9.2 ± 4.9	NBD	9.6 ± 2.9	NBD	39.9 ± 3.4	NBD	NBD	NBD	32.8 ± 15.2
43586/96	16.9 ± 1.2	NBD	4.6 ± 1.5	NBD	145.7 ± 59.7	NBD	NBD	NBD	56.2 ± 5.7
670/95	NBD	NBD	9.0 ± 5.5	NBD	206.2 ± 79.0	NBD	NBD	NBD	45.8 ± 26.3
39/2/14A	NBD	NBD	NBD	NBD	60.5 ± 23.1	NBD	27.9 ± 1.9	NBD	32.9 ± 12.4
S. constellatus									
48C	11.9 ± 3.9	NBD	NBD	NBD	119.5 ± 66.1	NBD	NBD	NBD	104 ± 26.3
34C	38.4 ± 21.2	NBD	28.5 ± 11.8	NBD	54.8 ± 25.8	NBD	NBD	NBD	82.6 ± 5.2
350/96	19.4 ± 5.8	NBD	40.6 ± 13.7	NBD	204.4 ± 135.9	NBD	26.6 ± 13.4	NBD	75.1 ± 14.6
F436	4.2 ± 1.1	NBD	7.7 ± 4.9	NBD	53.2 ± 23.3	NBD	11.2 ± 3.1	NBD	60.4 ± 13.1
322/95	775.6 ± 90.1	NBD	72.4 ± 24.9	NBD	439.5 ± 148.7	NBD	16.9 ± 5.1	NBD	283 ± 46.1
S. intermedius									
11C	134.4 67.1	NBD	102.9 ± 37.7	NBD	139.8 ± 72.9	NBD	31.9 ± 15.9	NBD	109.5 ± 17.2
30C	136 ± 17.2	NBD	1378.1 ± 284.7	NBD	1532.2 ± 114.0	74.3 ± 58.9	112.4 ± 50.1	NBD	1270.8 ± 115
84C	130.5 ± 40.2	NBD	266 ± 114.7	NBD	1133.7 ± 115.7	NBD	155 ± 8.1*	NBD	158.9 ± 23.5
127/95	168.1 ± 23.8	NBD	219.5 ± 91.5	NBD	258.1 ± 66.0	42.4 ± 3.9	NBD	NBD	452 ± 48.2
HW13	23.6 ± 14.9	NBD	29.8 ± 17.3	NBD	147.3 ± 63.3	25.0 ± 5	64.0 ± 13.1	NBD	251 ± 32.1
447/95	NBD	NBD	19.1 ± 8.9	NBD	184.6 ± 44.6	NBD	NBD	NBD	180.5 ± 23.1

*$p < 0.05$; Shaded boxes represent isolates recovered from healthy sites; Clear boxes represent isolates recovered from clinically infected sites; NBD = No binding detected.

with other strains binding to the same ligand). Pathogenic strains of *S. constellatus* showed higher adherence to alveolar bone and gingival proteoglycans compared with the commensal stains within the same subgroup. Generally, bacterial strains appeared to demonstrate no preferential binding to either recombinant decorin or biglycan ($p > 0.05$). Exceptions were seen for *S. constellatus* 322/95, which showed significant preferential adherence to recombinant decorin ($p < 0.001$), while *S. intermedius* 30C showed significant preferential adherence to recombinant biglycan ($p < 0.001$). Removal of the GAG chains from the respective proteoglycans abolished or significantly reduced bacterial adherence to the ligand ($p < 0.001$for all comparisons).

3.3. GAG depolymerisation activity

S. constellatus strains 322/95 and 34C and *S. intermedius* strains 127/95 and 30C were the only strains demonstrated to possess high chondroitinase and hyaluronidase activity, as demonstrated by an increase in 232 nm absorbance of 0.5 units or greater compared with no bacterial control (Table 1).

4. Discussion

An increasing number of microbes have been shown to depend upon extracellular matrix components for adhesion to host cells and tissues (Menozzi et al., 2002; Rostand and Esko, 1997; Wadstrom and Ljungh, 1999). Moreover, adhesion of microbes to host components has been proposed as the critical initial step in the establishment of bacteria as commensals or in the initiation of infection. Members of the SAG have previously been shown to adhere to the matrix components laminin, fibronectin and fibrinogen and weakly to collagens type I and IV (Allen et al., 2002; Willcox and Knox, 1990). In this study, we demonstrated the additional ability of the extracellular matrix proteoglycans decorin and biglycan to bind SAG, initiating the formation of microbial biolayers. Significantly, the results demonstrate that the interaction is mediated almost entirely by the GAG chains conjugated to the respective core protein.

SPR technology using the Biacore® 3000 system enabled the contribution of specific extracellular matrix components in the early events of biofilm formation in real time. The proteoglycan ligands represented either decorin or biglycan or a mixture of the two proteoglycans, and were confirmed to have been purified from other matrix components such as serum proteins and fibronectin. The C1 chip used in this study was devoid of dextran which, as a polysaccharide glucan, could have produced additional potential binding sites. Random covalent coupling was utilised to immobilise the proteoglycans at the surface of the sensor chip and to ensure the display of an ensemble of potential multivariant ligand orientations on the proteoglycan. This solid phase ECM adherence assay more closely mimics in vivo conditions, where matrix components are tethered. Sensograms obtained during the 250 s period indicated a fluctuating SPR signal. This could be considered comparable to the in vivo formation of biofilms, where models describe the initial reversible attraction of passively transported bacteria to the protein surface by long-range weak physicochemical forces, such as

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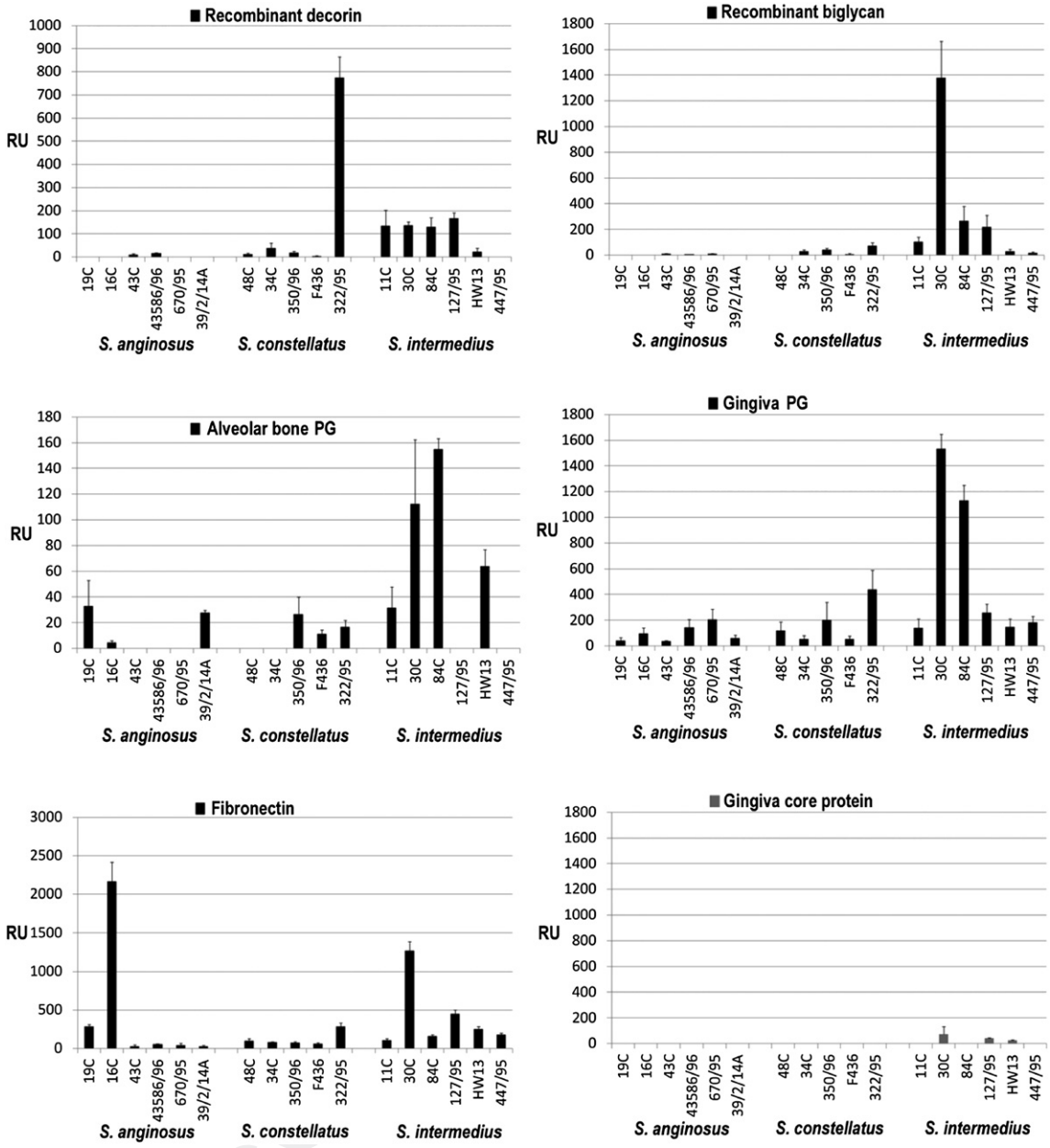

Fig. 4. Graphical comparison of adherence of different SAG isolates to the respective decorin and biglycan preparation. SAG isolates were analysed in triplicate and injected over the immobilised proteoglycan in a random order. Means and SD were determined. Whilst this method does not provide highly accurate values, it does allow identification of trends and gross changes in adherence patterns.

the van der Waals forces (Marsh and Martin, 2009). These attractions can be broken by net repulsive forces between the bacteria and the protein surface and by shear flow of liquid across the surface, which is mimicked within the Biacore 3000 system. Attachment subsequently becomes less reversible by stereochemical interactions forming between adhesins on the bacteria and proteins within the extracellular matrix. Generally, *S. intermedius* exhibited moderate to strong adherence to most proteoglycan ligands, whilst the majority of *S. anginosus* strains exhibited low adherence of less than 50 RU, particularly to the C4S-rich proteoglycans of recombinant decorin and biglycan and alveolar bone. There appeared to be no preferential adherence of commensal isolates compared to pathogenic isolates or vice-versa. As a positive control, all strains bound to fibronectin.

In this study, all SAG isolates were observed to adhere to gingival proteoglycans. For *S. intermedius* and *S. constellatus* strains, adherence was observed to be relatively strong and for isolates 670/95, 48C, 305/96, 322/95, 11C, 30C and 84C, adherence was statistically significantly greater than adherence to fibronectin. It is recognised that SAG may interact with a number of components within the extracellular matrix, such as laminin, fibronectin and fibrinogen (Allen et al., 2002; Willcox and Knox, 1990). However, the strong interactions observed comparable to fibronectin provide support for considering proteoglycans, particularly those in gingival

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tissues, as important constituents in facilitating SAG biofilm formation. Only 59% bound to alveolar proteoglycans and 70–75% bound to recombinant proteoglycans, with only *S. intermedius* 30C showing significantly greater adherence to recombinant biglycan compared to fibronectin ($p < 0.001$). Characterisation of the GAG chain demonstrated that a high proportion of ginigival proteoglycans were substituted with DS, whilst other proteoglycan ligands carried the C4S GAG chain. Removal of the DS or C4S chain with chondroitinase ABC abolished SAG adherence for the majority of isolates (adherence reduced to very low levels for three *S. intermedius* isolates). This suggests that the GAG chain plays a major role in adherence of bacterial isolates, and DS chains facilitate stronger binding compared with C4S chains. C4S and DS are both composed of a linear polysaccharide assembled as disaccharide repeat units containing *N*-acetyl-galactosamine and hexuronic acid that are linked together by β-glycosidic (β1,4 or β1,3) linkages. DS is structurally related to CS; however, a high proportion of uronic acid of DS is epimerised to iduronic acid, whilst CS contains mostly glucuronic acid. Casu et al. (1988) reported that epimerisation of uronic acid to iduronic acid provides additional degrees of rotation within the GAG chain. This additional flexibility is proposed to enable the negatively charged sulphate and carboxyl groups on the chain an easier search for sites on basic receptors on interacting molecules, thereby improving their adherence potential. Of note, iduronic acid is also present in significant amounts in heparin and heparin sulphate, which have also been reported to bind readily to streptococci isolates (Almeida et al., 1999a; Frick et al., 2003; Srinoulprasert et al., 2006; Tonnaer et al., 2006). Within this study, the interaction of SAG with the proteoglycan ligand was assessed at a physiological pH of 7.4. Increases or decreases in pH would lead to respectively increased ionisation or increased protonation of dissociable groups, on both the GAGs and the bacterial cell surface adhesins, the level of which depended upon the dissociation constant of the functional groups involved in facilitating the interaction. During inflammation, the pH of the gingival pocket can vary from pH 6.5 to 8.5 (Leblebicioglu et al., 1996), which could potentially influence GAG − bacterial adherence, although in the absence of any observable extremes in pH changes in inflamed tissues, these effects are likely to be small.

The nature of the bacterial adhesins on the SAG that facilitate binding to proteoglycans is unknown. Numerous matrix binding adhesins have been described in streptococcal species and, in general, fall into two groups (Lofling et al, 2010); the MSCRAMMs (microbial surface cell recognition adhesion matrix molecules) and a class of adhesins which have been reported to preferentially recognize sialylated glycoconjugates. Within the MSCRAMMs, M protein and protein F on the surface of *S. pyogenes* have been shown to adhere to DS, heparin sulphate and heparin (Frick et al., 2003), the alpha C protein adheres to heparin sulphate (Wang et al., 2011) and the FOG (fibrinogen-binding protein) binds DS and heparin sulphate in group G streptococci (Egesten et al., 2011). Outside of the streptococcus group of bacteria, decorin binding proteins (Dbp A, DbpB, Bgp) have been described in the spirochete *Borrelia burgdorferi* sensu lato group (Leong et al., 1998). The decorin binding site of DbpA has been mapped to a conserved peptide motif (EAKVRA), with binding mediated by the GAG chain, particularly DS (Salo et al., 2011). In those studies, DbpA showed considerably stronger binding under the flow conditions provided by SPR analysis, compared with stationary incubation adhesion assays. The authors hypothesise that the DbpA adhesion acts as a "catch bond" and sheer force is required to induce stronger interaction with the decorin (Salo et al., 2011).

The interaction between bacteria and the extracellular matrix is proposed to promote bacterial colonisation, facilitate bacterial invasion into deeper tissues and facilitate systemic dissemination. The adherence to extracellular matrix components may also be important in the pathogenesis of infection, although the precise involvement of extracellular matrix components such as decorin and biglycan is unclear. Binding of Group B streptococcal alpha C protein to host cells has been proposed to involve multiple binding to cell surface heparin sulphate proteoglycans, and the competitive inhibition of alpha C protein with exogenous GAGs promotes host cell survival and lowers the bacterial burden (Baron et al., 2009). Similar in vitro studies have demonstrated that cell surface GAGs facilitate the interaction of *Streptococcus pneumococcus* (Tonnaer et al., 2006), *S. uberis* (Almeida et al., 1999a) and *Streptococcus pyrogenes* (Frick et al., 2003) with host cells, which is interrupted by removal of the GAG by chondroitinases, GAG synthase inhibitors and exogenous GAGs. This may suggest that other GAGs within the extracellular matrix act as inhibitors of bacterial/host cell interactions. However, in providing a novel mechanistic viewpoint, studies have also suggested that GAGs may act as molecular bridges in directing binding to other matrix components such as vitronectin (Duensing et al., 1999). Most notably, preincubation of *S. uberis* with GAGs, particularly the iduronic acid-rich GAGs, enhanced beta-casein-mediated adherence to and internalisation in mammary epithelial cells (Almeida et al., 2003). In relation to the study reported herein, it is unclear at present whether binding of bacteria to the GAG chains of decorin or biglycan as an extracellular matrix protein would inhibit subsequent cell binding or facilitate it. Of note, selected isolates of *S. constellatus* and *S. intermedius* strains (322/95, 34C, 127/95, 30C) possessed high chondroitinase activity, which was absent from the more poorly-adhering *S. anginosus* isolates which might be important in release of the GAG chain from the parent molecule and subsequent interactions, be it as an enhancer or an inhibitor.

In conclusion, this study has demonstrated the ability of SAGs, particularly *S. constellatus* and *S. intermedius*, to adhere to the GAG moiety of the extracellular matrix proteoglycans decorin and biglycan. Adherence was greater for the DS proteoglycans of the gingival proteoglycan. This may be of significance in formation of dental abscesses, since viridians-group streptococci are frequently encountered deep inside the gingival connective tissue and associated periodontal ligament, which contains similar DS proteoglycans.

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The ability of isolates of *S. constellatus* and *S. intermedius* to adhere to C4S proteoglycans of alveolar bone may enhance their survival and potential virulence in the periapical region of a non-vital tooth, leading to infection and abscess formation. As with other bacterial ECM models, interactions formed with the extracellular matrix may be significant in influencing the metabolic activity of the host tissue and the phenotype/genotype of the bacterial cell, thus determining whether or not these "commensal" organisms initiate an infective process.

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