Archives of Oral Biology 82 (2017) 203–208

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Research Paper

Role of proteoglycans on the biochemical and biomechanical properties of dentin organic matrix

Cristina de Mattos Pimenta Vidal[a,b], Ariene Arcas Leme-Kraus[a], Momina Rahman[a], Ana Paula Farina[a,c], Ana K. Bedran-Russo[a,*]

[a] Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, 801 South Paulina St, Chicago, IL, 60612, USA
[b] Department of Operative Dentistry, College of Dentistry, University of Iowa, 801 Newton Rd, Iowa City, IA, 52242, USA
[c] School of Dentistry, University of Passo Fundo, BR 285, São José,Building A7, Passo Fundo, RS, 99052-900, Brazil

ARTICLE INFO

Keywords:
Dentin
Proteoglycans
Glycosaminoglycans
Ultimate tensile strength
Biodegradation

ABSTRACT

Objective: Proteoglycans (PGs) are multifunctional biomacromolecules of the extracellular matrix of collagen-based tissues. In teeth, besides a pivotal regulatory role on dentin biomineralization, PGs provide mechanical support to the mineralized tissue and compressive strength to the biosystem. This study assessed enzymatic protocols for selective PGs removal from demineralized dentin to determine the roles of these biomacromolecules in the bulk mechanical properties and biostability of type I collagen.
Methods: Selective removal of glycosaminoglycans chains (GAGs) and PGs from demineralized dentin was carried out by enzymatic digestion protocols using chondroitinase ABC (c-ABC) and trypsin (Try). A comprehensive study design included assessment of dentin matrix mass loss, biodegradability of the PGs/GAGs-depleted dentin matrix, ultimate tensile strength (UTS) and energy to fracture tests. Quantitative data was statistically analyzed by two-way and one-way ANOVA followed by the appropriate post hoc tests ($\alpha = 0.05$).
Results: Transmission electron microscopy images show effective GAGs removal by c-ABC and Try and both enzymatic methods released statistically similar amounts of GAGs from the demineralized dentin. Try digestion resulted in about 25% dentin matrix mass loss and increased susceptibility to collagenolytic digestion when compared to c-ABC ($p = 0.0224$) and control ($p = 0.0901$). Moreover, PGs digestion by Try decreased the tensile strengths of dentin. Statistically lower energy to fracture was observed in c-ABC-treated dentin matrix.
Conclusions: GAGs plays a pivotal role on tissue mechanics and anisotropy, while the core protein of PGs have a protective role on matrix biostability.

1. Introduction

Dentin is a mineralized tissue composed of 70 wt.% hydroxyapatite crystals and 20 wt.% organic extracellular matrix (ECM). The latter is mainly represented by type I collagen and small amounts of non-collagenous components (Goldberg, Kulkarni, Young, & Boskey, 2011; Linde & Goldberg, 1993). Dentin forms the bulk of tooth and thus is the main dental tissue affected in pathological conditions such as caries disease and mal-formations. The determination of functional roles of the extracellular matrix to the biomechanics and biostability of dentin could lead to the development of innovative biomimetic based preventive and restorative therapies. The functional roles of the mineral content and collagenous network has been largely investigated (Kinney, Habelitz, Marshall, & Marshall, 2003); however, only recently the contributions of non-collagenous components such as proteoglycans (PGs) on tissue's physical properties has gained attention.

PGs are glycosylated biomacromolecules formed by a core protein with one or more covalently attached glycosaminoglycan chains (GAGs), present in many collagen-based tissue including dentin, cartilage, skin and bone. PGs represent a small fraction of the dentin ECM while sustaining pivotal roles in the formation and arrangement of this tissue. GAGs are linear disaccharide chains that contain acidic sugar residues and/or sulfate groups that are negatively charged and can attract cations and/or water molecules (Prydz, 2015). Unlike cartilage, dentin holds mainly members of the small leucine rich proteoglycans (SLRPs), including chondroitin sulfate (CS)-rich decorin and biglycan, and keratan-sulfate (KS)-rich fibromodulin and lumican (Embery, Hall, Waddington, Septier, & Goldberg, 2001). A transition in the PGs profile from the pre-dentin to dentin and different GAG chains length confirm the existence of pools of PGs and different molecule structures attributed to their specific roles in dentin formation and mineralization (Goldberg et al., 2003; Waddington, Hall, Embery, & Lloyd, 2003).

* Corresponding author at: Department of Restorative Dentistry, UIC College of Dentistry, 801 South Paulina Street, Room 531, Chicago, IL, 60612, USA.
E-mail address: bedran@uic.edu (A.K. Bedran-Russo).

http://dx.doi.org/10.1016/j.archoralbio.2017.06.020
Received 11 November 2016; Received in revised form 11 May 2017; Accepted 14 June 2017
0003-9969/

PGs attract water molecules into the interfibrillar spaces of the dentin matrix regulating a hydraulic mechanical support system to the type I collagen network. The intricate interactions between GAG chains among PG and between PGs and collagen (Bertassoni, Orgel, Antipova, & Swain, 2012; Bertassoni & Swain, 2014) suggest that PGs interact with type I collagen by protein core binding to four or more collagen microfibrils via hydrogen bonds assuming a helical configuration. The GAG chain forms interfibrillar bridges by connecting adjacent collagen fibrils at specific intervals when wrapping around them in an anti-parallel position (Bertassoni & Swain, 2014; Bertassoni et al., 2012; Orgel, Eid, Antipova, Bella, & Scott, 2009). Those observations were confirmed in few studies on dentin (Breschi et al., 2003; Prati, Mazzoni, Nucci, Di Lenarda, & Mazzotti, 2007), but scarce reports are available on the specific roles of PGs and GAGs in the mechanical behavior of mineralized tissues such as dentin. More recently, their site contribution to the mechanical properties of dentin has been nanocharacterized in a creep deformation study after selective removal of PGs or GAGs (Bertassoni, Kury, Rathsam, Little, & Swain, 2015).

Correlations between an imbalance of PGs synthesis or concentration with tissue strength and biodegradability are reported in cartilage and bone (Leyh et al., 2014; Martel-Pelletier, Kwan Tat, & Pelletier, 2010), while it is unclear if PGs have any role in this aspect in dentin. Moreover, there is a lack of knowledge in the specific mechanisms involved in the participation of these biomacromolecules on the bulk tissue mechanical behavior and overall ECM biostability. The present study aimed to selectively remove PGs components to determine their roles on the biomechanical properties and biochemical stability of demineralized dentin matrices. Different enzymatic methods to remove GAGs and the core protein of PGs were used to determine the efficacy, specificity and effects on the ECM of dentin.

2. Materials and methods

2.1. PGs/GAGs removal quantification

Mid-coronal dentin was cut in a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under water irrigation to obtain 45 dentin specimens (1.5 mm × 1.5 mm × 0.5 mm) from extracted intact human molars (IRB # 2011-0312). All specimens were demineralized in 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 5 h under agitation (Castellan, Pereira, Grande, & Bedran-Russo, 2010). Specimens were then rinsed in distilled water (DW) and dried in desiccator for 24 h. The specimens were weighed on an analytical balance (XS105DU, Mettler Toledo Inc., Columbus, OH, USA), rehydrated in distilled water for 1 h and selective removal of PGs/GAGs was carried out as follows (n = 15): incubation in 0.67 U/ml chondroitinase ABC (c-ABC) from *Proteus vulgaris* (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.1 M Tris-Acetate pH 7.8 for 48 h at 37 °C under agitation or incubation in 1 mg/ml trypsin (Try) from bovine pancreas TPCK treated (Sigma-Aldrich) diluted in 0.2 M ammonium bicarbonate at the same conditions. Control group was incubated in DW. Solutions were replaced after 24 h of incubation. Then, specimens were washed in DW for 24 h in the same conditions and placed in desiccator to obtain the dry weight after PGs/GAGs removal. To determine the remaining amount of PGs/GAGs in dentin, specimens were re-hydrated in distilled water and incubated overnight with 50 μg/ml proteinase K (Thermo Scientific, Waltham, MA, USA) diluted in 100 mM K_2HPO_4 pH 8.0 at 56 °C (Barbosa et al., 2003).

The supernatant was collected and sulfated GAGs removal was quantified spectrophotometrically (Barbosa et al., 2003). Briefly, 100 μl of each solution was mixed with 1,9-dimethylmethylene blue (DMMB) (Sigma-Aldrich) solution to promote GAG complexation with DMMB. The complex was separated from the soluble material and solubilized with decomplexation solution. GAGs concentration was determined using a standard curve with different concentrations of chondroitin sulfate sodium salt from shark (Sigma-Aldrich) from 0.5 to 64 μg/ml. Absorbance was read at 656 nm in a spectrophotometer (Spectramax Plus, Molecular devices, Sunnyvale, CA, USA). Background subtraction was done using absorbance readings of the buffers used to dilute c-ABC and Try enzymes. GAGs concentration was normalized by the dry weight of dentin specimens before GAGs removal and expressed in μg/ml/mg of dentin. Statistical analysis was done using one-way ANOVA and Scheffe post-hoc test ($\alpha = 0.05$).

2.2. Biodegradability following PGs/GAGs removal

Dentin specimens were prepared as described above. After GAGs removal, dentin was washed and incubated with 100 μg/ml of collagenase from *Clostridium hystoliticum* (Sigma-Aldrich) in 0.2 M ammonium bicarbonate buffer (pH 7.9) for 24 h at 37 °C under agitation (Bedran-Russo, Castellan, Shinohara, Hassan, & Antunes, 2011) (n = 10). Collagenase solution was replaced after 1, 2, 4, 8 and 24 h, and every day until complete degradation of specimens (up to 7 days of incubation). Collagen solubilization was estimated by hydroxyproline (HYP) release at each time point using a method previously described (Reddy & Enwemeka, 1996) with minor modifications. In brief, lyophilized collagenase solution was re-suspended in 10 μl of DW and 40 μl of 2 M sodium hydroxide and hydrolyzed by incubation at 120 °C for 1 h. Then, solutions were incubated with chloramine T reagent for 25 min at room temperature followed by 1 M Ehrlich's reagent for 40 min at 65 °C. Standard curve was done using 2 to 25 μg/ml of *trans*-4-hydroxy-L-proline (Sigma-Aldrich). Absorbance was read at 550 nm in a spectrophotometer (Spectramax Plus). HYP release was calculated according to dry weight of the specimens and expressed in μg/ml/mg dentin. Total HYP release was statistically analyzed by one-way ANOVA and Scheffe post-hoc test ($\alpha = 0.05$).

2.3. PGs/GAGs removal evaluation by transmission electron microscopy

Freshly extracted molars were incubated with 10% buffered neutral formalin at 4 °C for 24 h. Demineralized dentin specimens were prepared as described above, stored in 10% buffered neutral formalin for 3 more days at 4 °C and PGs/GAGs removal was performed following the same protocols described above (n = 5). Then, dentin specimens were fixed with 25 mM sodium acetate pH 5.8 containing 2.5% glutaraldehyde for 30 min, washed with sodium acetate solution without glutaraldehyde for 30 min, stained with 0.05% cupromeronic blue (CB) (Sigma-Aldrich) in 25 mM sodium acetate containing 0.1 M $MgCl_2$ and 2.5% glutaraldehyde for 3 h at 37 °C (Bedran-Russo, Pereira, Duarte, Okuyama, & Yamauchi, 2008). Specimens were stained with 0.034 M sodium tungstate for 30 min and dehydrated in ascending concentrations of 200 proof ethanol (25%, 50%, 75%, 90%, 95% and 100%). Then, specimens were embedded in epoxy resin and sections of 70 nm were obtained and observed under transmission electron microscopy (TEM) (JEOL JEM-1220, JEOL Ltd., Tokyo, Japan).

2.4. Ultimate tensile strength and energy to fracture of PGs/GAGs-depleted dentin

Twenty sound molars had their roots removed and the crowns were sectioned in the occlusal-cervical direction into approximately 0.5 mm thick sections. The sections were further trimmed to obtain hour-glass shape slabs with 0.5 ± 0.1 mm at the mid-coronal dentin according to the tubule orientation [parallel (PL) and perpendicular (PP)] (Bedran-de-Castro, Pereira, & Thompson, 2004) (Fig. 1). Specimens were demineralized in 10% phosphoric acid for 5 h (Castellan et al., 2010) and further divided (n = 15) according to PGs/GAGs removal strategies described above [c-ABC, Try, and no removal (DW)]. The edge of the specimens were glued with a cyanoacrylate adhesive to a Ciucchi's jig mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) and tested in tensile at a crosshead speed of 1 mm/min. All specimens were kept hydrated in DW prior and during the tensile test.