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Isolation and Purification of Versican and Analysis of Versican Proteolysis

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Abstract

Versican is a widely distributed chondroitin sulfate proteoglycan that forms large complexes with the glycosaminoglycan hyaluronan (HA). As a consequence of HA binding to its receptor CD44 and interactions of the versican C-terminal globular (G3) domain with a variety of extracellular matrix proteins, versican is a key component of well-defined networks in pericellular matrix and extracellular matrix. It is crucial for several developmental processes in the embryo and there is increasing interest in its roles in cancer and inflammation. Versican proteolysis by ADAMTS proteases is highly regulated, occurs at specific peptide bonds, and is relevant to several physiological and disease mechanisms. In this chapter, methods are described for the isolation and detection of intact and cleaved versican in tissues using morphologic and biochemical techniques. These, together with the methodologies for purification and analysis of recombinant versican and a versican fragment provided here, are likely to facilitate further progress on the biology of versican and its proteolysis.

Keywords

Versican; Glycosaminoglycan; Extracellular matrix; Hyaluronan; Chondroitin sulfate; Affinity chromatography; A disintegrin-like and metalloprotease domain with thrombospondin type 1 motif; ADAMTS

1 Introduction

1.1 Versican and the Extracellular Matrix

Versican, also known as CSPG2 or PG-M, is a large chondroitin sulfate proteoglycan with a widespread distribution [1]. It is prominently expressed during embryogenesis, and is found in adult brain, cardiovascular system, skin, and musculoskeletal tissues [2–4]. Versican exists primarily as a large aggregate with the glycosaminoglycan hyaluronan (HA) [5], a property it shares with other members of the hyalactan (or lectican) family, which include aggrecan, neurocan, and brevican. In contrast to versican, these family members have a restricted distribution, with aggrecan primarily confined to cartilage, and neurocan and brevican selectively present in the central nervous system.

⁷In tissue sections, it does not automatically follow that immunostaining using GAG-domain antibodies solely indicates the presence of intact versican. Following proteolytic cleavage, the GAG domains will be contained in the C-terminal fragment and may linger owing to the multiple interactions of the G3 domains. The fate of versican fragments following proteolysis is not known.

¹⁴The versikine expression plasmid was previously described [38].

Versican has a well-established significance in biology, especially during embryogenesis and in the pathogenesis of several human diseases. Versican is crucial for myocardial and valvular development, and indeed, *Vcan* deficient mice die at mid-gestation as a result of cardiac anomalies [6–8]. Versican is implicated in neural-crest cell migration, and is required for musculoskeletal development [9, 10]. *VCAN* mutations that alter normal *VCAN* exon splicing in humans give rise to a rare eye disorder called Wagner syndrome, and a related condition named erosive vitreoretinopathy [11].

Versican has been extensively investigated in the context of acquired human disorders, specifically in cardiovascular disorders such as atherosclerosis and arterial stenosis [12, 13], and more recently, in a wide variety of cancers, where its presence is typically associated with increased tumor malignancy and metastasis [14, 15]. In the context of these disorders, a variety of cellular effects have been attributed to specific versican domains in vitro [16–18], although the specific cellular mechanisms for the observed effects remain to be fully elucidated.

Like the other hyalactans, the versican core protein has amino- and carboxyl-terminal globular domains (G1 and G3 respectively, *see* Fig. 1) [19]. The structure of the intervening region bearing the CS chains, however, depends on the splice isoform [19, 20]. The *VCAN* gene encompasses 15 exons, of which exon 7 and exon 8 encode large chondroitin sulfate-binding domains named GAG α and GAG β respectively. The splice isoform V2 contains only GAG α /exon 7, V1 contains only GAG β /exon 8, and V0 contains both; the smallest versican isoform, V3, has only the globular domains without an intervening CS-attachment region (Fig. 1). The G1 domain contains two link protein modules that mediate its interaction with HA [21, 22] (Fig. 1). This interaction may be further stabilized by the inclusion of link protein to form a trimeric complex. The HA-binding property of versican is relevant to its purification, because dissociation from HA is required as a preliminary step for isolation of the native proteoglycan. The CS-bearing region is a major contributor to the hydrodynamic properties of the HA–versican complex, and specific associations of the CS chains with cytokines (such as the C–C chemokine midkine, and TGF β) and elastin-binding protein have been identified [9, 23]. The strongly anionic nature of the CS chains is a characteristic that is exploited for versican purification by ion-exchange chromatography. The G3 domain is known to bind to the extracellular matrix proteins fibrillin-1, tenascin-R, and fibulin-1 and -2 [24–26]. Through these N- and C-terminal interactions, versican participates in specific extracellular matrix networks. The connection of HA to cell surface receptors or HA synthases renders versican an important component of pericellular matrix (PCM, also referred to as the glycocalyx) in several cell types such as fibroblasts, neurons (where PCM is also known as the perineuronal net), myoblasts, and smooth muscle cells [27–30]. For readers interested in additional details, the diverse interactions of versican have been reviewed elsewhere [31].

Although many proteinase classes may have catalytic activity toward the versican core protein, there has been considerable recent interest in ADAMTS proteases [32], which have been shown to attack specific peptide bonds, i.e., Glu⁴⁴¹-Ala⁴⁴² in the GAG β domain of the V1 isoform (the corresponding cleavage site numbering in human versican V0 is Glu¹⁴²⁸-Ala¹⁴²⁹) and Glu⁴⁰⁵-Gln⁴⁰⁶ in the GAG α domain of the V2 and V0 isoforms [33]. The

cleaved V1 N-terminal fragment (extending to Glu⁴⁴¹) is now referred to as versikine, and the corresponding V2 fragment (extending to Glu⁴⁰⁵) was first isolated as GHAP (Glial hyaluronic acid binding protein) [34]. The generation of cleavage site-specific antibodies, termed neo-epitope antibodies, which react only with ADAMTS-cleaved versican, but not the intact form [33], was instrumental in revealing the biological significance of ADAMTS-mediated versican proteolysis [35–39]. Because versican is a component of the provisional matrix of the developing embryo, its clearance is required as development progresses and specialized matrices replace the embryonic provisional matrix. In addition, as a component of PCM, it stands at the interface between the cell membrane and its microenvironment, and consequently, versican proteolysis can influence cell behavior. For example, the amount of PCM versican and the activity of the versican-degrading protease ADAMTS5 in PCM strongly influence the fibroblast to myofibroblast transition [28], a pivotal step not only in wound healing, but also in its pathological counterpart, fibrosis. ADAMTS genes can be upregulated at sites of versican turnover in a coordinated fashion [38]. Single or compound ADAMTS gene deletions in mice have established that versican proteolysis is required during interdigital web regression, cardiovascular development (including valve and myocardial development), melanoblast colonization of skin, craniofacial development (palatogenesis), myogenesis, and ovulation [30, 35–43]. The embryologic roles of versican proteolysis were the subject of a recent review [44]).

In addition to the inherent complexity of ECM, the unique attributes of proteoglycans, i.e., their extensive, frequently variable glycosylation, large size and highly anionic nature, complicate the analysis of versican. However, these properties are useful for devising purification schemes. The majority of studies of versican to date have focused on the versican V1 isoform and the globular domains. Much less is known about the other isoforms. A recently identified novel isoform, V4, is generated from a cryptic splice site in exon 8 (Fig. 1) and is hitherto known only as an RNA species [14]. Here, we provide methods for isolation of versican from tissues and cultured cells, as well as versican characterization and tissue localization. The protocols provided encompass expression and purification of recombinant truncated forms of versican, including versikine.

1.2 Detection of Versican and Its Proteolytic Fragments in Tissue Sections

The different versican isoforms have distinct expression sites in tissues. Furthermore, it is becoming apparent that the G1 domain-containing versican V1 proteolytic fragment, versikine, may mediate processes that are different to those of unprocessed versican. These differences highlight the importance of correctly identifying the different versican isoforms using biochemical and immunohistochemical/immunofluorescent approaches as outlined here.

1.3 Analysis and Quantification of Versican Content in Tissues and Cell Culture

Often, analysis of versican in tissues by immunofluorescence is not sufficient to accurately characterize the versican content of tissue samples or cultures. Instead, an alternative approach that identifies the molecular species by Western blot or a dual approach may be appropriate. Here, we describe the extraction and purification of versican and versikine from both cells and tissue samples and provide an approach that can be used to quantify the

extracted versican. Previously, methods have been described to isolate and purify versican from both animal and cell sources that used guanidine hydrochloride to disrupt versican interactions [8]. The present method uses urea as the chaotropic agent.

1.4 Expression and Purification of Recombinant Truncated Versican and Versikine

Methods for isolation of native versican from intact tissues and cell lines use tissue or ECM disruption with a chaotropic agent followed by ion-exchange chromatography, capitalizing on the highly anionic nature of the CS chains [21, 33, 45, 46]. Here, we describe methods for the expression and purification of recombinant versikine and V-5GAG (a truncated form of versican V1 that contains the G1 domain and the N-terminal-most five GAG chains, but lacks the G3 domain). These forms either do not exist in nature (V5-GAG) or may have low abundance in tissue (versikine) and yet, are useful for the analysis of versican processing and the biological impact of versican proteolysis respectively and are readily obtained in recombinant form. They are expressed and purified as recombinant proteins using Ni²⁺ NTA affinity for the (His)₆ tag as the first step, followed by HA-affinity purification.

2 Materials

2.1 Detection of Versican and Its Proteolytic Fragments in Tissue Sections

1. Citrate-EDTA antigen retrieval buffer: 10 mM citric acid, 2 mM EDTA, 0.05 % (v/v) Tween-20, adjust to pH 6.2.
2. PBS wash buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.1 % (v/v) Tween-20.
3. Vectashield or Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA).
4. Polyclonal rabbit anti-mouse versican antibody 1360–1439 (Anti-GAG β , EMD Millipore, Billerica, MA)—catalogue number AB1033.
5. Polyclonal rabbit anti-mouse versican antibody 535–598 (Anti-GAG β , EMD Millipore, Billerica, MA)—catalog number AB1032.
6. Polyclonal rabbit anti-human/mouse versican V0, V1 neo-epitope antibody (anti-DPEAAE, ThermoFisher Scientific, Waltham, MA)—catalog number PA11748A. This antibody specifically detects ADAMTS cleaved versican isoforms V0 and V1. The corresponding fragments are 1,428 and 441 amino acids in length.
7. Polyclonal rabbit anti-human versican V0, V2 neo-epitope antibody (anti-NIVSFE, ThermoFisher Scientific, Waltham, MA)—catalog number PA3-119. This antibody specifically detects the N-terminal fragment (GHAP) of ADAMTS cleaved versican isoforms V0 and V2.
8. Monoclonal mouse anti-human versican hyaluronate-binding region (Anti-G1, Developmental Studies Hybridoma Bank, Iowa City, IA)—catalog number 12C5.

9. Monoclonal mouse anti-human versican G3 domain (previously marketed by Seikagaku)—catalogue number 2B1.
10. Secondary antibodies are typically enzyme- or fluorophore- conjugated goat anti-rabbit IgG or goat anti-mouse IgG, as appropriate.

2.2 Analysis and Quantification of Versican Content in Tissues and Cell Culture

1. Extraction buffer 1: 8 M urea, 50 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 7.5.
2. Extraction buffer 2: 8 M urea, 50 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 8.0.
3. Wash buffer 1: 600 mM NaCl, 100 mM Na-acetate, 1 mM EDTA, adjust to pH 6.
4. Wash buffer 2: 50 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 8.0.
5. Elution buffer 1: 1.5 mM NaCl, 100 mM Na-acetate, 1 mM EDTA, adjust to pH 6.0.
6. Elution buffer 2: 500 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 8.0.
7. Q-Sepharose Fast Flow.
8. StrataClean Resin.

2.3 Expression and Purification of Recombinant Truncated Versican and Versikine

1. Polymeric hyaluronan (1.2 megaDalton HA. Hyalose, Oklahoma City, OK).
2. 1-ethyl-3-(3-dimethylaminot-propyl) carbodiimide.
3. Chondroitinase ABC lyase (C' ABC).
4. Dialysis Buffer 1: 4 M Guanidine-HCl (GuHCl), 100 mM NaH₂PO₄, 10 mM Tris-HCl, 10 mM imidazole, adjust to pH 8.
5. (His)₆ wash buffer 1: 100 mM NaH₂PO₄, 150 mM NaCl, 8 M urea, 20 mM imidazole, adjust to pH 8.
6. (His)₆ Wash buffer 2: 50 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, adjust to pH 8.
7. (His)₆ Elution buffer 2: 50 mM NaH₂PO₄, 500 mM NaCl, 250 mM imidazole, adjust to pH 8.
8. HA wash buffer 1: 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 7.5.
9. HA elution buffer: 4 M GuHCl, 50 mM Tris-HCl, 1 mM EDTA, adjust to pH 7.5.
10. Ni²⁺-NTA agarose.
11. EAH Sepharose-4B.

3 Methods

3.1 Detection of Versican and Its Proteolytic Fragments in Tissue Sections

3.1.1 Paraffin-Embedded Sections: Antigen Retrieval (See Note 1)

1. Remove paraffin by immersing slides in clearing agent (HistoClear or xylene) 2 × 5 min at room temperature (RT).
2. Rehydrate sections by successive immersion for 5 min at RT in 100 % EtOH (2×), 70 % EtOH, and dH₂O.
3. Place slides in a glass slide holder and fill with citrate/EDTA buffer. Place in microwave and heat at 500 W for 90 s, leave to stand for 30 s, and repeat heating step.
4. If necessary, top up the container with citrate/EDTA buffer to replace any lost through evaporation, leave to stand for 30 s, and repeat heating cycle (i.e., 90 s heat, 30 s stand, and 90 s heat).
5. Leave to stand in citrate buffer for 30 min to cool to room temperature.

3.1.2 Staining—If frozen sections are used, start the protocol from this step (see Note 2).

1. Block in 5 % (v/v in PBS) normal goat serum for 30 min at RT (see Notes 3 and 4).
2. Wash 3 × 5 min with wash buffer.
3. Add ~100 µl primary antibody (for concentrations see Table 1) for 1 h at RT or overnight (o/n) at 4 °C.
4. Wash 3 × 5 min with wash buffer.
5. Add secondary antibody, e.g., appropriately diluted enzyme/fluorophore-conjugated antibody and incubate at RT for 1 h (see Note 5).
6. Repeat step 4.
7. If using immunofluorescence, stain the nuclei by adding one drop of DAPI-containing mounting medium, seal and image (see Tables 2 and 3). Hematoxylin or another appropriate nuclear counterstain may be used for immunohistochemistry.

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¹The duration of fixation affects antigen availability and is thus an important variable that affects the results. It is recommended that samples that are to be compared to each other use identical fixation times in equivalent volumes of 4 % paraformaldehyde (PFA) at 4 °C to reduce variability.

²For sample comparison, all tissue samples should be processed identically (e.g., frozen sections will differ in signal intensity from paraffin-embedded sections).

³All immunostaining steps are performed in a humidified chamber to prevent evaporation and changes in the concentration of reagents.

⁴For secondary antibodies not raised in the goat, an alternative blocking agent is required that is specific to the species providing the secondary antibody. For example, if the secondary antibody was produced in the donkey, then normal donkey serum should be used to block.

⁵All antibodies described here were raised in mouse or rabbit. Secondary antibody is dependent on the primary antibody used, e.g., an antibody raised in mouse will require a secondary antibody specific to mouse.

8. Staining is evident in extracellular matrix (*see* Notes 6–8).

3.2 Analysis and Quantification of Versican Content in Tissues and Cell Culture

3.2.1 Extraction of Pericellular Versican from Cell Culture (See Note 9)

1. Culture cells of interest to ~80 % confluence, and wash with PBS (*see* Note 10).
2. Add serum-free medium and incubate for 24 h.
3. Remove serum-free medium and keep for further analysis.
4. Wash cells 3× with PBS.
5. Add 10 ml extraction buffer 1 for each 25 cm² cell culture plate, using a cell scraper to ensure that all cells are detached from the cell culture dish.
6. Adjust the volume to 30 ml with extraction buffer 1 and incubate on ice for 30 min, vortexing every 5 min.
7. Equilibrate 2 ml Q-Sepharose Fast Flow resin with 5 column volumes of extraction buffer 1.
8. Centrifuge extracted cells from **step 6** at 1,000 × *g* to remove large particulates/cell debris.
9. Combine resin with supernatant and mix by rotating o/n at 4 °C.
10. Transfer resin slurry to a sintered gravity flow column and collect flow-through.
11. Wash with 5 column volumes extraction buffer 1.
12. Wash with 5 column volumes wash buffer 1.
13. Elute bound protein with 5 column volumes of elution buffer 1, collecting each eluted fraction.
14. Take media from **step 3** and add urea to a final concentration of 8 M.
15. Combine with 2 ml Q-Sepharose Fast Flow resin equilibrated with 5 column volumes of extraction buffer 1.
16. Extract versican by repeating **steps 10–13**.

3.2.2 Extraction of Pericellular Versikine from Cell Culture (See Note 9)

1. Culture cells of interest to ~80 % confluence, and wash with PBS (*see* Note 10).
2. Add serum-free medium and incubate for 24 h.

⁶Controls are very important for tissue localization by antibodies. The primary antibody should be omitted from the protocol as a negative control. A mid-gestation mouse embryo section (gestational age 9.5–12.5 days) can be used as a positive control. Versican is abundant in the heart, brain, developing limb, and many other locations [2–4, 35–39]. A good positive control for anti-DPEAAE staining is the regressing interdigital web (e.g., forelimb autopod from a 13.5 old embryo) [38].

⁸Examples of immunostaining with versican antibodies or neo-epitope antibodies can be obtained in the literature [4, 35–38, 42, 43]. Pretreatment of sections using chondroitinase ABC treatment may improve antibody binding.

⁹Protein extracts should be processed immediately and kept on ice to prevent degradation.

¹⁰The amount of versican produced by isolated primary cells of different origins is likely to vary and so this step will require optimization for each cell type used. Typically 25 cm² monolayer cultures will yield sufficient versican for analysis and quantification.

3. Remove serum-free medium and keep for further analysis.
4. Wash cells 3× with PBS.
5. Solubilize cells in 10 ml extraction buffer 2 for each 25 cm² cell culture plate, using a cell scraper to ensure all cells are detached from the cell culture dish.
6. Equilibrate 2 ml Q-Sepharose Fast Flow resin with 5 column volumes of extraction buffer 2.
7. Centrifuge extracted cells from **step 5** at 1,000 × *g* to remove large particulates/cell debris.
8. Combine resin with supernatant and mix by rotating o/n at 4 °C.
9. Transfer resin slurry to a gravity flow column and collect flow-through.
10. Wash with 5 column volumes extraction buffer 2.
11. Wash with 5 column volumes wash buffer 2.
12. Elute bound protein with five column volumes of elution buffer 2 collecting each eluted fraction.

3.2.3 Extraction of Versican or Versikine from Tissue Samples—As described above for cell culture extraction, the extraction of versican or versikine from tissue is carried out using extraction, washing, and elution buffers 1 and 2 respectively.

1. Homogenize tissue sample in 1:10 (w/v) of tissue to the appropriate extraction buffer (e.g., 10 mg tissue added to 1 ml buffer) on ice using a tissue homogenizer. Ensure that the weights of the samples being compared are identical prior to homogenization.
2. Increase the volume of extraction buffer 1 fivefold and incubate on ice for 30 min, or overnight at 4 °C for tough tissues such as tendon, vortexing every 5 min for 30 s.
3. Centrifuge at 10,000 × *g* for 10 min to remove large particulates.
4. Equilibrate 0.2 ml Q-Sepharose resin with either extraction buffer 1 or 2.
5. Combine equal volume of supernatant from **step 3** with the equilibrated Q-Sepharose resin.
6. Rotate o/n at 4 °C.
7. Transfer to a sintered gravity flow column and collect flow-through.
8. Wash resin with 5 column volumes of extraction buffer followed by 5 column volumes washing buffer.
9. Elute bound protein with 5 column volumes of elution buffer collecting all fractions.

3.2.4 Quantification of Versican/Versikine

1. For versican analysis incubate each elution fraction with C'ABC at a final concentration of 0.1 U/ml at 37 °C for 3 h. C'ABC digestion is not required for versikine analysis (*see* Note 11).
2. Add 15 µl StrataClean to each fraction and mix by rotating at RT for 30 min (*see* Note 12).
3. Centrifuge for 5 min at 10,000 × *g*.
4. Discard supernatant and resuspend beads in SDS-PAGE loading buffer (*see* Note 12).
5. Add 2-mercaptoethanol (10 % v/v) and/or dithiothreitol to a final concentration of 0.1 M. Heat at 100 °C for 5 min and analyze by either 8 % or 10 % SDS-PAGE (for versican and versikine respectively) alongside versican protein standards (either versikine (Fig. 2) or V-5GAG as appropriate).
6. Perform Western blot using an antibody specific to the desired region (G1 domain, neo-epitope, or GAGβ).
7. Quantify extracted protein by measuring the band intensity of the loaded standards and drawing a standard curve. Use this curve to quantify the extracted protein amount (*see* Fig. 2).

3.3 Expression and Purification of Recombinant Truncated Versican and Versikine

3.3.1 Expression (See Notes 13–15)

1. Stable cell lines expressing versikine (residues 1–441 of human versican) and V-5GAG (residues 1–695 of human versican) are obtained using zeocin selection and expanded to ~500 cm² cell culture area (*see* Notes 15 and 16).

¹¹Without prior C'ABC digestion, versican, like other CS-proteoglycan, migrates as a smear at the very top of the gel. Its significantly retarded mobility within the gel results from the high negative charge and large size of the CS chains. These smears are most unlike the sharp bands typically seen on Western blots of unmodified proteins, and may puzzle observers new to proteoglycan analysis; moreover, they are often not reproducible between successive Western blots, may not transfer uniformly, and a significant proportion may be retained in the stacking gel. Upon C'ABC digestion, the CS chains are removed and the proteoglycan migrates as a better-defined band and the smear is eliminated or greatly attenuated. A mixture of unmodified core protein, and variable modification explain the dispersion of species that constitute the smear. The expected molecular mass for versican V0, V1, and V2 is 372, 265, and 182 kDa respectively, but the observed migration is typically at >350 kDa for V0 and V1, and at ~200 kDa for V2. Versikine has a predicted molecular mass of 49 kDa, but typically migrates at 70 kDa for reasons that are not presently understood [33, 38]. GHAP has a predicted molecular mass of 43 kDa, but migrates as a 64 kDa band [34].

¹²StrataClean resin binds all protein and can be used to extract protein from dilute samples for SDS-PAGE analysis (i.e., instead of protein precipitation). If multiple analyses are required, fractions should be aliquoted prior to addition of StrataClean resin. The resin does not need to be washed prior to use and can be added directly from the supplied container. The ethanol will be sufficiently diluted so as to not affect versican/versikine binding. The resin should be loaded directly onto the gel since the bound protein is not removed by SDS-sample buffer (i.e., it needs to be electrophoresed off) [22].

¹³The V1 construct in a pSecTagA plasmid [5] contained the entire open reading frame (ORF) but originally had an intervening 3'-untranslated sequence between the stop codon and the epitope tags. Therefore, an Xho I restriction site was inserted to disrupt the stop codon using the QuikChange Mutagenesis kit (Stratagene, Santa Clara, CA), the 3'-untranslated sequence was excised, and the vector religated to render the versican ORF continuous with the myc and (His)6 tags. To generate the V-5GAG construct with C-terminal myc and His6 tags, another Xho I site was placed at the appropriate location within the versican ORF. Mutagenized plasmid was digested with Xho I and the region between the two Xho I sites was separated by agarose electrophoresis followed by religation of the plasmid.

¹⁵It is important that all reagents used are sterile and that sterile technique is used throughout. Incubation steps are carried out at 37 °C and in 5 % (v/v) CO₂.

2. At ~70 % confluence, remove cell culture medium, wash cells with PBS, and replace with serum-free medium.
3. Incubate cells for 48 h.
4. Remove serum-free medium and store it at -80°C until used for protein purification, replace with medium containing 10 % (v/v) fetal calf serum, and incubate cells for 12 h (*see* Note 17).
5. Remove medium, wash cells with PBS, and replace with serum-free medium.
6. Incubate for 48 h and collect the medium, this constitutes a second collection.

3.3.2 Purification: Hyaluronan (HA) Affinity Column Preparation (to Make 40 ml HA-Affinity Resin)

1. Resuspend 10 mg polymeric hyaluronan (1.2 MDa HA) in 10 ml H_2O o/n at 4°C .
2. Wash 40 ml EAH-Sepharose 4B resin with 250 ml H_2O .
3. Add the resin to the HA, bring the volume to 90 ml with H_2O , and adjust pH to 4.7.
4. Add 0.4 g 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide to the HA resin slurry and stir at RT while readjusting the pH to 4.7 for 3 h (*see* Note 18).
5. Allow to stand o/n at RT.
6. Add 2 ml glacial acetic acid to block unsubstituted groups on the resin.
7. Wash sequentially with 500 ml 1 M NaCl, 500 ml 0.05 M formic acid, 500 ml H_2O , and 500 ml 0.5 M Na-acetate containing 0.02 % (w/v) Na-azide (*see* Note 19).
8. Store the HA-affinity resin in 0.5 M Na-acetate containing 0.02 % (w/v) Na-azide at 4°C .

3.3.3 Purification: Stage 1—His₆-tag Affinity Purification

1. Concentrate the collected serum-free conditioned medium to ~50 ml using concentrators with a 10 kDa molecular weight cut-off (*see* Note 20).

¹⁶Ensure selected stable lines are expressing sufficient protein before expanding colonies. Generally speaking, if expressed protein can be seen clearly on a Coomassie blue stained SDS-PAGE gel of the unconcentrated conditioned medium, expression levels are sufficient to achieve a good final protein yield (~2–3 mg/l). Proteoglycans do not take a Coomassie blue stain and may be stained using silver staining or toluidine blue.

¹⁷This step can often allow sufficient cell recovery to induce the production of more protein; however, this may not always be possible. Collected serum-free medium containing the expressed protein should be processed immediately (being kept on ice between procedures) or frozen until purification to prevent degradation.

¹⁸Carbodiimide reactions cause a gradual increase in pH, which will result in a progressive inhibition of the coupling reaction.

¹⁹It is often desirable to keep all eluted fractions to quantify the amount of HA bound to the column (thus making it possible to estimate the binding capacity of the column). This can be achieved using the meta-hydroxybiphenol reaction [47].

²⁰Any large-scale protein concentrating system can be used. For example Vivaflow 200 blocks (Membrane 5000PES, Vivascience Ltd).

2. Place into dialysis tubing (e.g., SnakeSkin, Pierce) and dialyze extensively against dialysis buffer 1 (*see* Note 21)
3. Equilibrate 5 ml Ni²⁺ NTA resin with 5 column volumes (i.e., 5 × 5 ml) of dialysis buffer 1.
4. Add the resin to the dialyzed conditioned medium and rotate at 4 °C o/n.
5. Transfer slurry to a sintered gravity flow column and collect flow-through.
6. Wash with 5 column volumes of (His)₆ wash buffer 1.
7. Wash with 5 column volumes of (His)₆ wash buffer 2 (*see* Note 22).
8. Elute with 5 column volumes of (His)₆ elution buffer 1, collecting 1 column volume fractions (Fig. 3a).
9. Dialyze washes and supernatant from **steps 5 and 6** into dialysis buffer 1 and repeat **steps 3–8**.
10. Digest elution fractions with 0.1U/ml chondroitinase ABC lyase (C'ABC) at 37 °C for 3 h, analyze by 10 % SDS-PAGE, and pool all fractions containing the purified proteins (Fig. 3) (*see* Note 23).

3.3.4 Purification: Stage 2—HA-Affinity Purification

1. Dialyze eluted fractions containing protein from Subheading 3.3.3 **steps 8 and 9** extensively against HA wash buffer 1.
2. Equilibrate 5 ml of HA resin with 5 column volumes HA wash buffer 1.
3. Combine dialyzed protein with HA-affinity resin and rotate at 4 °C o/n.
4. Transfer slurry to a sintered gravity flow column and collect flow-through.
5. Wash resin with 5 column volumes of HA wash buffer 1.
6. Elute bound protein with 5 column volumes HA elution buffer 1, collecting 1 column volume fractions (Fig. 3b).
7. Dialyze each elution fraction into HA wash buffer 1.
8. Take a 30 µl sample from each elution fraction and assess for protein content and purity by analysis using 10 % SDS-PAGE and visualize with Coomassie Brilliant Blue stain.

²¹This buffer has threefold actions: It partially unfolds the protein to ensure that all target protein is free of hyaluronan and it reduces nonspecific protein–protein interactions to reduce impurities. It also exposes the His-tag, improving binding to the nickel resin.

²²This step is necessary to remove all chaotropic agent and refold the protein.

²³Samples containing V-5GAG will require C'ABC treatment prior to SDS-PAGE analysis. After purification it is important to characterize the protein to ensure that the protein is functional and has the correct molecular weight. A solid phase hyaluronan-binding assay and mass spectrometry can be used [22].

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Abbreviations

ADAMTS	A disintegrin-like and metalloprotease domain with thrombospondin type 1 motif
CS	Chondroitin sulfate
ECM	Extracellular matrix
GAG	Glycosaminoglycan
HA	Hyaluronan
PCM	Pericellular matrix

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Fig. 1.

A schematic showing the known versican isoforms and the modular structure of its N-terminal (G1) and C-terminal (G3) domains. Versican interacts with HA via its G1 domain and forms interactions with other ECM components via its G3 domain. There are two epidermal growth factor (EGF)-like repeats in this domain, the lectin-like module resembles C-type lectins, and the complement regulatory protein (CRP)-like module is also referred to as a complement control protein (CCP) module

Fig. 2.

Illustration of a method to quantify unknown versican (versikine) samples using known standards. Versikine standards are run alongside unknown samples (a). The standards are then used to construct a standard curve (b), which can be used to derive total protein content in the samples using the gradient of the line (i.e., $y = mx + c$). This technique is possible because the antibody epitopes are identical in both the samples

Fig. 3. Coomassie blue stained gel showing versikine elution fractions. Purification from Subheading 3.3.3, **step 8** and Subheading 3.3.4, **step 6** (i.e., Ni^{2+} -NTA affinity and HA-affinity) are shown in (a) and (b) respectively. Elution is complete by fraction 3 in both steps

Table 1

Antibody concentrations used for versican detection

Antibody ^a	Concentration (µg/ml) for IHC	Concentrations (µg/ml) for WB
AB1033	2	0.5
AB1023	2	0.5
PA11748A	1–2 ^b	0.5
12C5	3	1
2B1	3	1

^aCatalogue number (see Subheading 2)^bFor paraffin-embedded sections use 2 µg/ml, for frozen sections use 1 µg/ml

Table 2

Versican domains detected by each antibody

Antibody ^a	Epitope position			
	G1 ^b	GAGα ^b	GAGβ ^b	G3 ^b
AB1033			x	
AB1023		x		
PA3-119		x		
PA11748A			x	
12C5	x			
2B1				x

^aCatalogue number (*see* Subheading 2)^bDomain structure shown in Fig. 1

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Table 3

Versican isoform recognized by each antibody

Antibody ^a	Isoform detected					Versikine ^c	GHAP ^d
	V1 ^b	V0 ^b	V2 ^b	V3 ^b			
AB1033	x	x					
AB1023			x				
PA3-119							x
PA11748A						x	
12C5	x	x	x	x	x	x	
2B1	x	x	x	x	x		

^aCatalogue number (see Subheading 2)

^bIsoform structure shown in Fig. 1

^cVersikine is the G1 domain containing ADAMTS-cleaved versican V1 fragment (N-terminus to E⁴⁴¹)

^dGHAP (glial hyaluronic acid binding protein) is the ADAMTS-cleaved N-terminal fragment of versican V0 or V2 (N-terminus to E⁴⁰⁵ inclusive)