Assay Development and Application for the Determination of Percent Modification of Divinyl Sulfone Modified Hyaluronan Hydrogel

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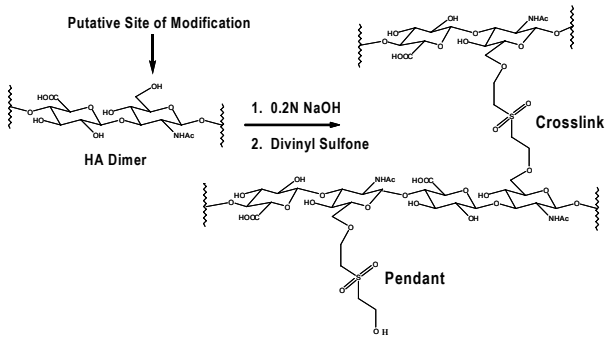
Introduction

Hyaluronan (HA) and its chemically modified derivatives have long been proven to be beneficial for treatment of pain due to osteoarthritis, prevention of post-surgical adhesions, drug delivery and cosmetic applications. Hylan B, a divinyl sulfone (DVS) modified HA, is a sterile, nonpyrogenic, viscoelastic, transparent gel composed of cross-linked HA molecules. DVS is a symmetrical bi-functional molecule, in which one or both vinyl groups can react with the hydroxy groups of the polymer to give a pendant group or a cross-link, respectively. In order to determine the type of modification (pendant vs. cross-link, see Scheme 1) and the total percent modification of HA, a method of enzymatic digestion followed by HPLC analysis was developed.



Scheme 1: Divinyl sulfone modified HA structure

By utilizing the assay, we not only gained a better understanding of the chemistry of the DVS modified HA, we were also assured of characterization of our gel production. More significantly, the assay provided information on the total percent modification and the relative contributions of pendant and cross-link moieties.

Materials and Methods

DVS modified HA gels were prepared by a variety of methods.[1, 2] *Streptomyces* hyaluronidase was purchased from CalBiochem. A CarboPac PA100 LC column was purchased from Dionex Corp. All gels were first diluted with 100mM sodium acetate, pH 5.0 buffer solution to a final concentration of 0.4% to ensure an optimal digestion condition. Then the divinyl sulfone modified HA gel was treated with *streptomyces*-derived hyaluronidase to undergo a 3-step hyaluronidase digestion. Theoretically, upon complete digestion, the enzyme should generate two oligosaccharide products from the natural substrate: a tetrasaccharide and a hexasaccharide with delta 4, 5-unsaturation at the non-reducing end.[3] Therefore, a digested fragment equivalent to an octasaccharide or bigger would indicate the presence of cross-linked modification. By employing the charge and size sensitive LC column, and optimizing the mobile phase composition, this specific HPLC method allowed distinct separation between tetramer, hexamer, octamer and decamer and so forth up to a 16-unit oligomer. Moreover, the generation of the 4, 5-unsaturated double bond offers UV detection at 232 nm for the assay. In an HPLC chromatogram of a test gel, any fragment that eluted between tetramer and hexamer peaks was identified as tetramer plus, representing the pendant-type DVS modification of HA. Any fragment that eluted between hexamer and octamer peaks was identified as hexamer plus, which also represented a pendant-type HA modification. However, all peaks eluted after the octamer peak were grouped as the cross-linked fragments. The relative percent modification of each type (i.e., pendant or crosslink) was determined based upon the area percentage of the peak representing each type of modification.

Results and Discussion

Gels, made under differing conditions, were evaluated by this assay and the results are listed in the table below.
It was observed that the total % modification of HA increased as the molar ratio of DVS: HA increased. In all cases, the increase in total % modification was a result of the increase from both pendant and cross-link modifications. In addition, the assay results demonstrated the high reproducibility of the assay and the tight control of our gel production.

Sample ID	RXN Description	Pendant %	X-linked %	Total % Mod.
Gel# A	0.15Meq DVS, ppt*	2.57	2.35	4.91
Gel# B	0.18Meq DVS, ppt*	3.51	2.64	6.14
Gel# C	0.21Meq DVS, ppt*	3.65	3.16	6.80
Gel# D	0.25Meq DVS, ppt*	4.49	3.85	8.33
Gel# E	0.30Meq DVS, ppt*	5.43	3.90	9.33
Gel# F	0.75~0.80Meq DVS	11.13	10.96	22.09
Gel# G	0.75~0.80Meq DVS	10.67	11.29	21.96
Gel# H	0.75~0.80Meq DVS	11.60	11.58	23.18
Gel# I	0.75~0.80Meq DVS	10.61	10.82	21.43
Gel# J	0.035Meq DVS	0.80	2.73	3.53
Gel# K	0.035Meq DVS	0.85	2.60	3.45
Gel# L	0.035Meq DVS	1.09	2.58	3.67

* ppt - precipitate

References

1. Balazs, et al. United States Patent 4582865 April, 1986
2. Balazs, et al. United States Patent 4636524 Jan., 1987
3. Chun, et al. , Analytical Biochemistry 171, 197-206, 1988