HYALURONAN

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EFFECT OF METAL IONS ON THE RHEOLOGICAL FLOW PROFILES OF HYALURONATE SOLUTIONS

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ABSTRACT

The effects of various metal ions on the rheological flow profiles of hyaluronate solutions were investigated, by controlled incubation of metal chloride salt solutions with sodium hyaluronate (NaHA) solution under ambient conditions. Results from application of the Williamson model to the flow profiles of incubated solutions showed a progressive decrease in Williamson zero shear viscosity (η_0) with increasing atomic number / atomic mass of the metal ion, which, with the exception of data for Li^+, could be related to a power equation ($y = cx^b$). Such reductions in viscosity were not a result of hydrolysis (determined by GPC), but may be due to diffusing ions disrupting hydrogen bonding and shielding electrostatic repulsions between carboxylate groups.

INTRODUCTION

Hyaluronan is a biocompatible / biodegradable, linear, water-soluble, ionic polymer composed of repeating (1→4) linked disaccharide units consisting of β-D-Glc*p*A and β-D-Glc*p*NAc, linked together by (1→3) glycosidic bonds [1]. It has a high molecular weight (around 10^5-10^7 Da), depending on source, giving a DP range of ~ 250-25000 [2]. It is found in all vertebrates, being present in almost every tissue as a component of the extracellular matrix and is distributed throughout the mammalian body, especially in synovial fluid, loose connective tissue, umbilical cord and the vitreous body of the eye [3]. The largest amount of hyaluronan (7-8 g per average human, 50 % of the total in the body) is in the skin tissues (both the dermis and epidermis) [4]. The most commonly isolated / utilised (*in vitro*) forms of hyaluronan are the free acid (hyaluronic acid, HA) and its sodium salt (sodium hyaluronate, NaHA).

In solution the hyaluronan backbone is stiffened by the chemical structure of linked disaccharide units, internal hydrogen bonds, mutually repelling anionic groups and solvent interactions, making it a rigid and highly hydrated molecule. It adopts an expanded random coil structure in physiological solutions, occupying a large domain. Small molecules, e.g. water and electrolytes, can freely diffuse through the domain, whilst large molecules are partially excluded due to their hydrodynamic size. At low concentrations, individual chains entangle forming a continuous network, giving viscoelastic and pseudoplastic properties, which is unique for a water-soluble polymer at low concentration. At higher concentrations entangled networks can be formed, which can resist rapid, short duration fluid flow, thus exhibiting elastic properties. However, short fluid flow of longer duration can partially separate and align molecules, allowing movement and thus exhibiting viscous properties [5].

The viscoelastic properties of hyaluronan solutions are ideal for use as a biological shock absorber and lubricant, which is why it is present in synovial fluid, where it lubricates the cartilage between joints. The cartilage provides a cushion between the bones allowing the joint to move smoothly. However, in an arthritic joint the elasticity / viscosity of the joint fluid is reduced, diminishing the shock absorbing and barrier properties [6]. Highly viscoelastic hyaluronan solutions can be injected into joints (viscosupplementation) in order to restore the rheological environment of the joint and thus improve joint function. This is used in osteoarthritic joints to provide instant protection and shock absorption, thus decreasing pain associated with mobility.

The aim of this investigation was to see how the interaction of hyaluronan with a range of metal ions (of differing ionic size, valency, etc.) affected the rheological flow characterisitics (especially the zero shear viscosity, η_0) and molecular weight profile. This is of particular interest with respect to localised *in vivo* applications of hyaluronan, since there are numerous metal ions in the body which could interact with administered hyaluronan resulting in significant changes in desired physical properties.

MATERIALS & METHODS

Preparation of boiled, nitrogen flushed, deionised water

Deionised water was boiled (~ 5 minutes, under vacuum) to remove dissolved air, cooled to ambient temperature, and flushed with nitrogen (~ 15 minutes) to displace any residual air, and prevent air redissolving. The resultant boiled, nitrogen flushed, deionised water was refridgerated (4 °C) until required, and was used for the preparation of all subsequent solutions (the NaHA and metal salt solutions detailed below). Boiling / nitrogen flushing was used to exclude dissolved oxygen from the water to try and minimise any oxidative hyaluronan degradation effects.

Preparation of sodium hyaluronate solution

Sodium hyaluronate (NaHA, 6 g, produced by microbial fermentation using *Streptococcus equi*) was dispersed in boiled, nitrogen flushed, deionised water (600 mL) in a conical flask (1 L), and the flask headspace was flushed with nitrogen. The flask was stoppered and the NaHA allowed to dissolve slowly over a period of ~ 48 hours (at 4 °C), with ocasional gentle agitation / swirling to assist solubilisation, resulting in a viscous, homogenous NaHA solution (1 % w/v). Care was taken to avoid the use of any metal equipment in the production of the hyaluronate solution (especially items containing iron / stainless steel), to minimise potential degradatory effects.

Preparation of metal ion solutions

Metal chloride salt solutions (0.25 M) were prepared by dissolving the necessary amounts of anhydrous lithium chloride (LiCl, M_w 42.39), sodium chloride (NaCl, M_w 58.44), potassium chloride (KCl, M_w 74.55), anhydrous calcium cloride ($CaCl_2$, M_w 111.0), manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$, M_w 197.9), cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$, M_w 237.9), and cerium (III) chloride heptahydrate ($CeCl_3.7H_2O$, M_w 372.6), in boiled, nitrogen flushed, deionised water (50 mL) in volumetric flasks (50 mL). All salt solutions were filtered (using 0.45 µm pore size, 25 mm diameter Titan nylon membrane filters), and flushed with nitrogen (to remove any dissolved air / oxygen), before use in hyaluronate incubation experiments.

Incubation of hyaluronate with metal ions

Aliquots of NaHA solution (1 % w/v, 5 mL) were transferred into individual headspace vials (20 mL, Merck). Aliquots of the metal chloride salt solutions detailed above (0.25 M, 5 mL) were transferred into the individual headspace vials containing the NaHA solution (in duplicate) to give overall concentrations of 0.5 % w/v NaHA and 0.125 M metal ions. The pipette tip used for individual NaHA solution dispensing was also utilised for subsequent metal ion solution dispensing, so that the latter ensured any residual NaHA was washed out of the pipette tip. Duplicate controls were also prepared using boiled, nitrogen flushed, deionised water aliquots (5 mL). The resultant solutions were flushed with nitrogen (to remove any dissolved air / oxygen, which also facilitated mixing), and the vials sealed with butyl/PTFE lined (3.0 mm) plain aluminium crimp caps (20 mm diameter, Merck). The solutions were incubated at ambient temperature, and the rheological flow profiles determined exactly 1 hour after metal ion solution addition (as detailed below). [This incubation test procedure had been previously validated by performing replicate rheological analyses on test solutions (NaHA and metal salts), which resulted in Williamson infinite shear viscosity (η_0) values with % variation values of < 10 %].

Determination of rheological flow profiles

All rheological flow profile measurements were performed using a TA Instruments AR 1000 'Rheolyst' controlled stress rheometer, equipped with 'Rheology Solutions' software (v. 1.2.2). The software is split into two modules, the 'AR1000' module controls the instrument itself and enables the operator to set up experimental procedures and perform the actual experiments, whilst the 'Data' module manipulates and presents the collected data. Duplicate rheological flow profile measurements were performed using the test parameters detailed below. Approximately 5 mL of sample was used for each flow test (the remainder being stored at 4 °C until molecular weight determinations were performed). The 'Data' module was used to apply the Williamson model to the resultant flow profiles (shear rate *vs* viscosity) in order to determine the Williamson zero shear viscosity (η_0) values. The Williamson model describes the low shear viscosity behaviour and is derived from the Cross model when $\eta >> \eta_\infty$ [7].

Geometry:	parallel plate (4 cm diameter)
Geometry gap:	500 μm
Geometry inertia:	~ 1.5 μNms^2 (calibrated before each test)
Instrument inertia:	~ 14.2 μNms^2 (calibrated daily)
Temperature:	20 °C (controlled by Peltier plate)
Pre-experimental shear stress:	1.768 Pa (for 10 s)
Test stress range:	0.5 – 500 Pa (log ramp)
Number of Points:	31 (max. point time 1 min)

Determination of molecular weight profiles

The molecular weight profiles of incubated solutions (0.5 % w/w NaHA, 0.125 M metal salt, & controls) detailed above were determined ~ 12-24 hours after incubation, by GPC analysis using the isocratic HPLC system detailed below.

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