Sodium hyaluronate of defined molecular size for treating osteoarthritis

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Sodium hyaluronate has been widely used in the treatment of osteoarthritis of the knee. In this communication we present the process of preparation of sodium hyaluronate of tailored molecular size with desired physico-chemical characteristics from bulk natural sodium hyaluronate, where the bulk material is obtained by extracting it from rooster combs. The physico-chemical properties of the bulk sodium hyaluronate and its various tailored fractions were studied. The bulk sodium hyaluronate was hydrolysed by steam under pressure, with variations in the process parameters, to attain the desired molecular size. The size-reduced sodium hyaluronate in the form of 1% solution in phosphate buffer had excellent rejuvenating effects when administered as intra-articular injection in osteoarthritis. More than 1500 patients suffering from osteoarthritis of the knee have been treated with satisfactory results.

Keywords: Knee and joint, osteoarthritis, sodium hyaluronate, viscosupplementation.

OSTEOARTHRITIS is characterized by a loss of articular cartilage, which has a limited capacity to heal by itself. Along with the cartilage changes, a reduction in the elastic and viscous properties of the synovial fluid occurs. The loss of visco-elasticity decreases the lubrication and protection of the joint tissue from attrition and this is one postulated mechanism of pain production in osteoarthritis. The concentration of hyaluronic acid reduces to almost half the normal in osteoarthritic knees[1]. Viscosupplementation can be achieved by injecting sodium hyaluronate into the affected joint[2]. Sodium hyaluronate is a linear polysaccharide consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl D-glucosamine (Figure 1). The use of sodium hyaluronate in patients with osteoarthritis relies partly on the premise that supplementation of synovial fluid with exogenous product substantially recovers[3,4] the diminished lubricating and cushioning properties of the endogenous substance seen in osteoarthritis. Sodium hyaluronate also influences the invasion process of cells participating in the acute and chronic inflammatory activities. A direct analgesic effect has been shown in a rat model[4,5]. Under shear stress, sodium hyaluronate acts as a lubricant, protecting adjacent joint surfaces from mechanical damage. Under load-bearing stress, sodium hyaluronate acts as a shock absorber, protecting the cartilage from compressive trauma. It also helps in cartilage nutrition by allowing small molecules such as water, electrolytes and nutrients to diffuse freely, whereas proteins and other inflammatory mediators encounter a higher degree of retardation in passing through its network[4,6].

In the chronic inflammatory processes of tissues, sodium hyaluronate can also be used as a vehicle for any kind of intra-articular medication to protect the effect of the drug by decreasing its diffusion out of the articular space. In all types of intra-articular surgery, sodium hyaluronate can be used to protect the articular cartilage surfaces from post-operative injury.

The average molecular weight of sodium hyaluronate derived from rooster combs ranges between 1.7 and 2 million Daltons (MDa). The molecular weight of sodium hyaluronate was determined using Mark–Houwink equation. The material used as such in the form of about 1% solution or the modified (chemically cross-linked) material of increased molecular weight produced from the above material to treat osteoarthritis gives rise to certain adverse reactions like injection-site pain, temporary pain, swelling, and/or fluid accumulation in the injected knee. There are also mild reactions like rashes, itching, cramping of the calves, hemorrhoids, muscle pain and lower-back sprain. These reactions may disappear after sometime even without treatment in most recipients. However, about 20% of the recipients cannot use these injections more than once or twice due to severe inflammatory reactions at the site of application. These aspects have been documented profusely in the literature[7–11]. It has also been documented that injection of lower-molecular weight sodium hyaluronate has been more effective than osmo equivalent saline or sodium hyaluronate of larger molecular weight[4]. Saline is isotonic with the body fluid and has no curative effect.

Bulk sodium hyaluronate can be converted into large molecular size polymer: the natural polymer has several reactive groups such as the primary alcoholic group, the carbamide group, the carboxylic acid and a host of secondary alcoholic moieties. Generally, the primary alcoholic groups have been utilized to form dimers, trimers and even tetramers, using formaldehyde or gluteraldehyde as the bridging materials. Polymerization has been carried out *in situ*, using the cock's combs as the starting material, followed by extraction of the high-molecular weight polymers; such polymerized materials have been mixed with low-molecular weight polymers to obtain aqueous solutions of desired viscosity[12].

Cock's combs were collected from slaughterhouses through contracts to enable the collection and immediate preservation of the combs from the sacrificed animals in appropriate conditions (clean plastic polypropylene bags containing ice) and to deliver the materials within 24 h at the processing premises so that these could be preserved at –70°C for longer periods. All the chemicals including acids, alkalis,

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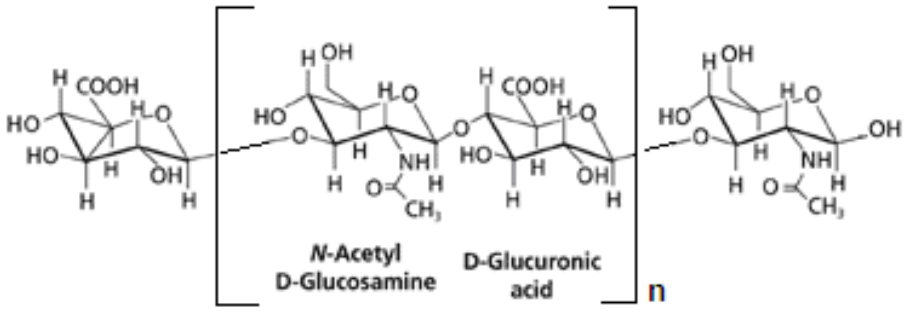

Figure 1. Chemical structure of hyaluronic acid polymer.

common salt, sodium phosphates, acetone, mannitol, chloroform, etc. were purchased from reputed suppliers. Ethyl alcohol denatured with acetone was procured through government permit from the Excise and Prohibition Department, District Mehsana, Gujarat. The process involves the preparation of sodium hyaluronate of defined molecular size and physico-chemical characteristics from bulk natural sodium hyaluronate; bulk sodium hyaluronate was prepared by extraction from rooster combs using the method of Balazs with certain modifications[13]. The process essentially consisted of cutting the rooster combs into small pieces with stainless-steel chopper and thereafter, the cut pieces were washed by immersing in ethanol : chloroform mixture (95 : 5 v/v). The ratio between cut pieces and solvent was 1 : 1 w/w. The mixture was stirred for about 4 h in 2 to 3 repeat cycles till the solvent layer was colourless. The solvent was completely decanted, leaving the washed tissues in the container. The extraction solution which is essentially water containing some additives like Mannitol (about 1500–2000 ppm) was used in the ratio of 3 : 1 (w/w) several times under low stirring condition (50–60 RPM) at room temperature (25°C). A minimum of 5–7 days was required to extract the sodium hyaluronate contents to the maximum extent. The aqueous extract was separated from the extracted rooster pieces by centrifugation at 3500 *g*. The aqueous extract was treated with sodium chloride to obtain a concentration of 10% w/v. An equal volume of chloroform was then added to the above solution. The mixture of two liquids was stirred to ensure proper contact between chloroform and the aqueous phase (50–60 RPM). Nearly 15–17 times the extraction was carried out using fresh solvents. The spent chloroform was then used in distillation. The chloroform layer removes most of the impurities present in the aqueous extract. The extraction is facilitated for removing the impurities by changing the pH from 4 in the beginning to 8 at the end. The aqueous extract then becomes almost free from all the impurities including proteins, endotoxins, nucleic acids and lipids. The solution was membrane filtered at 0.2 μm and the pure sodium hyaluronate was precipitated by contacting the solution with rectified spirit used in the proportion of 1 : 2 (v/v). The precipitated sodium hyaluronate was washed with pure acetone and dried under low humidity (RH < 25%) to a constant weight. One kilogram of rooster comb yields 1.2–1.4 g of dried sodium hyaluronate.

The physico-chemical properties of the whole sodium hyaluronate extracted from rooster combs were studied. The bulk was dissolved in phosphate buffer (pH 7.2–7.4) at concentrations of 0.5, 1 and 2% (w/v). Each solution was subjected to depolymerization by heating with steam at different temperature and time. The depolymerized solution from each aliquot was treated with rectified spirit to precipitate out the converted polymer, which was isolated by filtration and dried at low humidity condition (RH < 25%) at 25°C to a constant weight.

It was observed that a fraction of molecular weight between 1.0 and 1.3 MDa on re-dissolution into phosphate-buffer solution had a viscosity between 10000 and 30000 centipoises. This solution also behaved like a viscoelastic fluid as was evidenced by the decrease in viscosity on application of increased shear rate/pressure and the increase in viscosity, when shear rate was decreased. Fractions of molecular weight lower than the range of 1.0–1.3 MDa had diminished viscoelastic properties.

The change in viscosity of the natural polymer (mol wt 1.7–2 MDa), when subjected to increase/decrease in shear rate is given in Table 1.

The initial viscosity at a particular shear rate remains high and stabilizes after the spindle of the Brookfield viscometer attains 3–4 rpm.

The target molecular size of sodium hyaluronate was achieved after considerable variation in the process parameters. The best method was evolved by exposing sodium hyaluronate solution of molecular weight 1.7–2 MDa,

Table 1. Change in viscosity of 1% solution of bulk sodium hyaluronate in phosphate buffer solution, read on Brookfield viscometer at 25°C

Shear rate (s^{-1})	Viscosity range (Centipoise)
0.009	450000–120000
0.021	188000–108000
0.047	108000–90000
0.084	72000–63300

dissolved in isotonic phosphate buffer saline (pH 7.2 to 7.6) to saturated steam at positive gauge pressure (0.9 to 1.1 lb per sq. inch) in the temperature range of 100–130°C for a predetermined time ranging from 20 to 90 min.

The method of reducing the molecular weight of sodium hyaluronate requires depolymerization reaction, which is best conducted in about 1% aqueous phosphate buffer solution. The advantage of this process lies in the capability of controlling the molecular size quite precisely under the hydrated state than when carried out under anhydrous condition. In this context, Swann *et al.*[14] have reported that there is reduction in molecular weight of bulk sodium hyaluronate when the latter in the solid state is subjected to heating by steam for sterilization purposes. They have shown that molecular size reduction is substantial when sterilization is carried out in solution phase and therefore, they have claimed a sterilization process of the bulk sodium hyaluronate in the solid phase to be more desirable. They have not mentioned utility of sodium hyaluronate sterilized and size reduced in the solution phase by steam heating.

In our study solutions of sodium hyaluronate in phosphate buffer (pH 7–7.5) have been degraded under varying conditions of steam temperature ranging from 100 to 130°C, particularly at 121°C. Experiments were conducted to observe the effect of treatment at 121°C with varied time periods on molecular weight and viscosity of typically three different 1% sodium hyaluronate solutions (Tables 2 and 3). The data show the change in average molecular size with time for a 1% sodium hyaluronate solution heated at 121°C (Table 2). The change in viscosity with time for the above material under similar conditions is given in Table 3. In these experiments, the material was isolated by precipitation using rectified spirit in two volumes of alcohol to 1 volume of treated material. The material was isolated by filtration, dried at (< 25% RH) to a constant weight and reused for experimental measurements after dissolving in phosphate saline buffer. Figure 2 shows the effect of treatment at 121°C with varied time periods on viscosity of bulk sodium hyaluronate converted into 1% solution in phosphate saline buffer. It is apparent that more the time of treatment, more the decrease in viscosity.

Experiments were conducted utilizing 0.5, 1 and 2% solution of sodium hyaluronate in phosphate buffer to analyse the effect of initial concentration of sodium hyaluronate on treatment under varied time periods (min) at 121°C (Figure 3 and Table 4). It can be seen from Table 4 that 1% solution subjected to hydrolysis for 20 to 40 min provided the product with desired property, i.e. viscosity nearly between 10,000 and 30,000 centipose. The average molecular weight obtained varied from 1.17 to 1.3 MDa. Our experience with the use of higher-molecular weight bulk between

Table 2. Effect of treatment at 121°C with varied time periods on molecular weight of typically three different 1% sodium hyaluronate solutions

	Molecular weight (MDa)		
Time (min)	Exp. 1	Exp. 2	Exp. 3
0	2.0	1.7	1.66
20	1.71	1.12	1.1
40	1.36	1.03	1.0
60	1.03	0.64	0.6

Table 3. Effect of treatment at 121°C with varied time periods on viscosity of typically three different 1% sodium hyaluronate solutions. Sodium hyaluronate used in experiments 1–3 had initial molecular weights of 2.0, 1.7 and 1.66 MDa respectively. (Viscosity measured with Brookfield viscometer at 0.08 shear rate and 25°C)

	Viscosity (Centipoise)		
Time (min)	Exp. 1	Exp. 2	Exp. 3
0	200000	100000	90000
20	55556	22222	22000
40	19444	12778	12222
60	9444	2222	1667

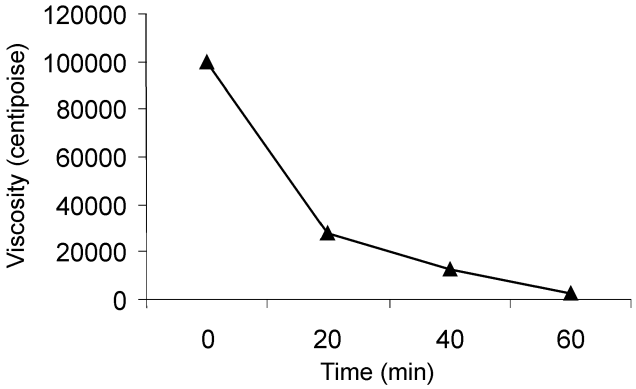

Figure 2. Effect of treatment at 121°C with varied time periods on viscosity of 1% sodium hyaluronate solution.

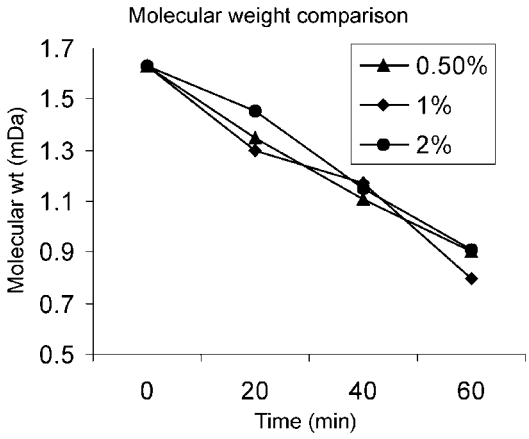

Figure 3. Molecular weight of solutions with differing initial concentrations treated for varied time periods.

Table 4. Effect of initial concentration of sodium hyaluronate on treatment under varied time periods (20, 40, 60 min) at 121°C

	Control		20		40		60	
% Solution	Visc.	Mol. wt	Visc.	Mol. wt	Visc.	Mol. wt	Visc.	Mol. wt
0.5	555.6	1.63	–	1.35	–	1.11	–	0.9
1	100000	1.63	27778	1.3	13333	1.17	2778	0.8
2	556000	1.63	280000	1.45	102000	1.15	31667	0.91

Mol. wt, Molecular weight in MDa. Visc., Viscosity in centipoise (measured with Brookfield viscometer at 0.08 shear rate and 25°C).

Table 5. Molecular size reduction of 1% solution of sodium hyaluronate at 100, 110 and 121°C for different time periods (20, 40, 60 min)

	121°C		110°C		100°C	
1% Solution	Visc.	Mol. wt	Visc.	Mol. wt	Visc.	Mol. wt
0	100000	1.63	100000	1.63	100000	1.63
20	27778	1.3	68333	1.57	77222	1.6
40	13333	1.17	66111	1.43	67222	1.47
60	2778	0.8	55556	1.39	57223	1.4

Mol. wt., Molecular weight in MDa. Visc., Viscosity in centipoise (measured with Brookfield viscometer at 0.08 shear rate at 25°C).

Table 6. Therapeutic effect of 1% sodium hyaluronate solution obtained by depolymerization on osteoarthritis patients

				Pain at rest and on movement at joint (based on VAS[#])	
Age	Sex	Knee injected	Osteoarthritis condition (Stage)	Before therapy	At the end of study
55	F	Right	III	Severe pain	No pain
53	F	Left	III	Severe pain	No pain
48	F	Left	III	Severe pain	No pain
33	F	Left	III	Severe pain	No pain
49	F	Left	III	Severe pain	No pain
39	F	Right	III	Severe pain	No pain
42	M	Left	III	Severe pain	No pain
48	M	Left	III	Severe pain	No pain
72	M	Right	III	Severe pain	No pain
48	F	Left	III	Severe pain	No pain

[#]VAS, Visual Analogue Scale.

1.66 and 2.0 MDa shows reduction in molecular weight up to 1 MDa after 40 min (Table 2). The initial molecular weight plays a role in attaining the desired size reduction within the optimum period.

In order to find out the optimum temperature and time period for depolymerizing sodium hyaluronate 1% solution, experiments were conducted at 100, 110 and 121°C for different time periods. It can be seen from Table 5 that hydrolysis of 1% solution of sodium hyaluronate in phosphate buffer at 121°C between the period of 20 and 40 min gave the product of the desired specification, i.e. viscosity between 10,000 and 30,000 centipoise and average molecular weight between 1.0 and 1.3 MDa.

The molecular weights of all the grades of sodium hyaluronate were determined using the standard Mark–Houwink equation given below.

$$\text{Intrinsic viscosity } [\eta] = KM^a,$$

where $[\eta]$ is the intrinsic viscosity, K and a are constants which changed with the solvent used to dissolve the polymer, the nature of the polymer and the temperature of determining viscosity and M is the molecular weight of the polymer.

$[\eta]$ was determined at 25°C using 0.2 M aqueous NaCl solution as the solvent from a plot of $(\ln \eta_r/C)$ vs C, where C is the concentration of sodium hyaluronate in g/ml and η_r is the relative viscosity. The intercept gives $[\eta]$. Using this and the values of K and a from the literature[15], given as $K = 2.28 \times 10^{-4}$ and $a = 0.816$, we calculate the molecular weight of each fraction of sodium hyaluronate.

One per cent solution of sodium hyaluronate of average molecular weight between 1.0 and 1.3 MDa, in phosphate

buffer and with viscosity between 10,000 and 30,000 centipoise in pre-filled syringes was produced under license from the Drugs Control authorities and injected into knees of people suffering from osteoarthritis. The results are given in Table 6. It can be seen from Table 6 that the formulation had excellent rejuvenating effect on all the recipients. Moreover, none of the patients had any noteworthy inflammatory reaction. After the completion of the efficacy studies, more than 4500 sterile, pre-filled syringes were made available to more than 1500 patients suffering from osteoarthritis of the knee. The results of application have been excellent.

Sodium hyaluronate of molecular weight between 1.0 and 1.3 MDa in the form of 1% solution in phosphate buffer, with a viscosity between 10,000 and 30,000 centipoises behaves like a viscoelastic fluid. It shows excellent therapeutic effects on all recipients suffering from osteoarthritis of the knee. More than 4500 pre-filled syringes were made available to about 1500 patients from all over the country.

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Extension of range of distribution of *Nasikabatrachus sahyadrensis* Biju & Bossuyt (Amphibia: Anura: Nasikabatrachidae) along Western Ghats, with some insights into its bionomics

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***Nasikabatrachus sahyadrensis* Biju & Bossuyt is observed to occur in the forested habitats of the Nilgiri ranges north of Palakkad Gap, indicating its extension of range of distribution from the hitherto known limit south of the Palakkad Gap between 8 and 11°N lat, i.e. Anamalai and Cardamom Hills in the Western Ghats. The adult individual of *N. sahyadrensis* freshly unearthed from a scooping pit in the habitat was tested for its behavioural tendencies for burrowing in the field and feeding in the laboratory under captive conditions in a glass tank filled with damp soil and prey. Some aspects of bionomics of this fossorial frog focusing on its burrowing and underground foraging behaviours with reference to morphological and ethological characteristics of the taxon are presented here.**

Keywords: Bionomics, burrowing, foraging behaviour, *Nasikabatrachus sahyadrensis*, Palakkad Gap.

BIJU and Bossuyt[1] described the burrowing frog *Nasikabatrachus sahyadrensis*, a new amphibian species from India, veritably acknowledged by animal taxonomists and biogeographers all over the world as one of the rarest kinds of 'once in a century find'[2]. Molecular dating estimates and phylogenetic DNA analyses of the frog, recognized under a new family, indicate the new taxon's relationship with frogs of the family Sooglossidae endemic to Sey-

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