Synthesis and characterization of a novel double crosslinked hyaluronan hydrogel

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Hyaluronan has great potential in medicine as a biomaterial. However, in its native form, hyaluronan is rapidly metabolized *in vivo* by free radicals and enzymes such as hyaluronidase, and it is highly soluble. Various methods have been adopted therefore, to modify the physicochemical properties of hyaluronan, while maintaining biocompatibility, and thereby widen its spectrum of therapeutic applications.

Hyaluronan has four reactive groups (acetamido, carboxyl, hydroxyl and the reducing end) available for crosslinking to itself or other polymers. Using a variety of crosslinking agents, researchers have developed a host of crosslinked hyaluronan derivatives with an increased *in vivo* residence time. This chemical modification has enabled the production of gels and films, which can be used in applications such as the prevention of post-surgical adhesions, wound healing and dermal augmentation.

We have found that if hyaluronan is crosslinked to itself, or to other polymers (either synthetic or biopolymer), in two stages, then a high degree of crosslinking is achieved, conferring improved biostability. In each of the two stages, the same crosslinking agent is used, but different functional groups are bound by altering the reaction conditions. The novel process can be tailored to yield water insoluble gels and films with a broad range of physical and chemical characteristics, and greater resistance to degradation by hyaluronidase and free radicals. These derivatives are currently undergoing biocompatibility testing, and should ultimately lead to a series of innovative second-generation medical products.

1. Introduction

Hyaluronan (hyaluronic acid; sodium hyaluronate), abbreviated as HA, was first discovered by Meyer and Palmer in 1934 [1]. Hyaluronan is a long linear polysaccharide comprising repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid. The molecule has unique rheological properties, which allow it to behave as a viscoelastic gel even at low concentrations.

HA is widely distributed throughout the body and forms the capsule of some bacterial species. It is found in particularly high concentrations in the vitreous humor of the eye, synovial fluid of joints and the umbilical cord. As a general constituent of the extracellular matrix, HA in combination with collagen, chondroitin sulfate and other glycosaminoglycans is able to support cell and tissue growth. The viscoelasticity and the hydrophilic nature of hyaluronan enable it to retain the tonicity and elasticity of the tissue in which it is incorporated. In addition it acts as a lubricant when present in body fluids and can maintain the shape of a body cavity when compromised. These physicochemical properties coupled with the biocompatibility and non-immunogenic nature of the molecule enable its use in a wide range of clinical applications.

As a biocompatible hydrogel, HA has received increasing attention for use in the controlled delivery of drugs [2], wound healing [3], prevention of post-surgical adhesions [4], the correction of dermal deformities such as depressed scars and wrinkles, and for the augmentation of soft tissues [5]. HA is bioresorbable and is easily degraded *in vivo* due to the action of the enzyme hyaluronidase and free radicals. This restricts its use in some medical applications, which require a long-lasting effect [6]. Therefore, in order to increase the residence lifetime of HA to extend its potential applications it is essential to modify HA whilst retaining its biocompatibility.

Modification of HA can be achieved via chemical modification. This can be divided into chemical derivatization and chemical crosslinking. Partial or total esterified HA can be produced by chemical derivatization of the carboxyl group of the polymer with an alcohol [7]. Through such modification, the residence time of the

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material is increased and the properties can be tailored by the degree of esterification [8]. Chemical crosslinking is commonly used to produce strong hydrogels, using crosslinkers such as divinyl sulfone [9], polyepoxide [10, 11], and glutaraldehyde [12]. The biostability and other physical properties of crosslinked HA have been improved by crosslinking the hydroxyl groups on the HA polymer. The use of water-soluble carbodiimide has also been reported to crosslink HA through reaction between the carboxyl and hydroxyl groups of HA or available amino groups [13, 14]. HA has been reported to react with synthetic polymers and biopolymers to form conjugates [15].

Epoxide is one of the most commonly used crosslinkers for biopolymers due to its high reactivity and well-achieved performances in biostability and mechanical properties. The crosslinking of HA has been achieved using a range of epoxides including 1,4-butanediol diglycidylether (BDDE) [16], 1,4-bisepoxybutane [10], and polyethlene glycol diglycidyl ether [11]. The functional groups, which are available to react with epoxide, are carboxyl and hydroxyl groups. In acidic conditions, the epoxide will react with carboxyl groups to form an ester linkage whilst an ether linkage will be formed under alkaline conditions. The proportions of ester-linkage and ether-linkage formed are dependent mainly on the pH value of the reaction medium. At pH $>$ 10, the linkages formed are ether-linkages whilst the ester-linkage is formed predominately when pH is lower than 8, which in turn alters the biodegradability of the final gel [17].

It was found that a crosslinked HA network can be built through crosslinking between both carboxyl/carboxyl and hydroxyl/hydroxyl groups of HA or HA/polymer combinations, which is called double crosslinking technology [18, 19], as shown in Fig. 1. According to this process, in the first step, stable ether-linkages are obtained by crosslinking through hydroxyl groups. The second step involves the synthesis of additional ester-linkages by crosslinking through carboxyl groups. We selected other polymers such as non-ionic synthetic polyvinyl alcohol (PVA) and ionic biopolymer sodium alginate, for combination with HA to perform our double crosslinking technology. This novel process has resulted in the generation of HA and HA/polymer derivatives with improved biostability.

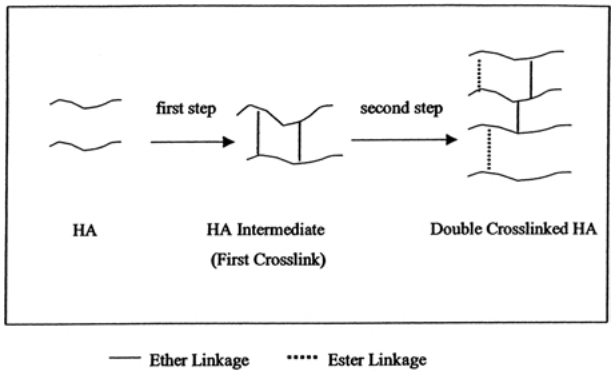

Figure 1 Scheme of novel process of double crosslinked HA.

2. Experimental

2.1. Synthesis of crosslinked HA

HA solutions between 1.5% to 5% in 0.5 M NaOH were prepared. Crosslinking agent epichlorohydrin or 1,2,7,8-diepoxyoctane with a hydrophobic methylene ''bridge'' was added and the reaction was carried out in a petri dish. After 72 h drying in a fume cupboard at room temperature, a dry sheet-like material was obtained. The dried sheet was neutralized with HCl solution and purified by washing the film with acetone/deionized H_2O (7/3(v/v)), IPA/ deionized H_2O (7/3(v/v)), and IPA. Thus the first crosslinked HA intermediate was obtained. The intermediate was subjected to a second crosslinking step by suspending it in acetone/HCl (7/3(v/v)) at pH $<$ 4.0. To this, crosslinking agent 1,2,7,8-diepoxyoctane was added. The crosslinking conditions are shown in Table I. After crosslinking for 24 h, the product was washed with IPA, IPA/deionized H_2O (7/3(v/v)), IPA and acetone. The samples were dried to obtain a constant weight.

2.2. Synthesis of crosslinked PVA/HA (CPH)

A 1% HA solution and a 5% PVA aqueous solution were prepared respectively. The solutions were mixed together to give a homogeneous HA/PVA solution with varied HA compositions as depicted in Table II. The solution was cast in a petri dish and dried in a fume cupboard for four days. The resulting film was suspended in a mixture of $CHCl_3$/acidic or alkaline solution/crosslinker (1,2,7,8-diepoxyoctane or glutaraldehyde). The crosslinking reaction was performed at room temperature for a fixed time period of 24 h. A further amount of crosslinking agent was added, and if necessary the pH was adjusted. The second crosslinking reaction was allowed to proceed for 24 h at room temperature. The detailed crosslinking conditions are shown in Table II. After crosslinking, the samples were washed with IPA and acetone three times and immersed into IPA/deionized H_2O(3/2(v/v)) overnight at room temperature. The film was finally washed with acetone and dried in an oven at 37 °C until a constant weight was obtained.

2.3. Synthesis of crosslinked HA-alginate film

A 2% sodium alginate aqueous solution was mixed with a 1% HA in phosphate buffered saline to give a homogeneous HA/Alginate solution. 10 ml of the above homogeneous solution was cast into a petri dish and calcium chloride solution added. This was left for complexation to occur. The resulting HA/alginate slab was washed three times with deionized H_2O to remove any unbound calcium. The slab was allowed to dry in a fume cupboard for 72 h. The resulting film was suspended in a solvent/alkaline solution and 1,2,7,8-diepoxyoctane was added with the reaction allowed to proceed at room temperature. The resulting film was washed with IPA/deionized H_2O, suspended in acidic solvent and a further volume of crosslinker 1,2,7,8-diepoxyoctane added. The reaction was allowed to proceed at room temperature for a fixed time. The detailed crosslinking conditions are shown in Table III. The crosslinked film was washed with acetone/deionized

TABLE I Formulation of crosslinked HA (CHA)

Sample	First crosslink				Second crosslink			
	Crosslinker	Feeding ratio	Time (h)	pH	Crosslinker	Feeding ratio	Time (h)	pH
CHA-1	E-1	0.375/1	72	OH^-	/	/	/	/
CHA-2	E-1	0.375/1	72	OH^-	E	0.5/1	24	H^+
CHA-3	E	0.375/1	72	OH^-	/	/	/	/
CHA-4	E	0.375/1	72	OH^-	E	0.5/1	24	H^+

E-1: epichlorhydrin (Aldrich); E: 1,2,7,8-diepoxyoctane (Aldrich); Feeding ratio: the weight ratio of HA to crosslinker. All reactions were performed at room temperature.

TABLE II Formation of crosslinked PVA-HA (CPH)

Samples	HA (%)	Crosslinker 1st/2nd	pH 1st/2nd	Time 1st/2nd (h)	WAC (%)
CPH-2	10	E/E	OH^-	24/24	280
CPH-3	10	E/E	H^+/OH^-	24/24	340
CPH-4	10	E/E	OH^-/H^+	24/24	250
CPH-6	20	G/E	H^+	24/24	600
CPH-7	20	E/E	H^+/OH^-	24/24	580
CPH-8	20	E/E	OH^-/H^+	24/24	480
CPH-13	50	E/E	OH^-/H^+	24/24	258
CPH-1	10	G	H^+	24	300
CPH-5	20	G	H^+	24	Dissolved
CPH-9	30	G	H^+	24	Dissolved
CPH-10	50	G	H^+	24	Dissolved
CPH-11	50	E/E	OH^-	24/24	3117
CPH-12	50	E/E	H^+	24/24	1084

E: 1,2,7,8-diepoxyoctane; G: glutaraldehyde; H+ represents a pH of about 4; OH^- represents a pH of about 10; CPH-1, 5, 9, 10, 11, 12 were all prepared using a single crosslinking step for comparative purposes; WAC(%): Water absorption capacity (%).

TABLE III Formation of double-crosslinked Alginate/HA (CAH)

Samples	HA (%)	Crosslinker 1st/2nd	Ca^{2+} Conc^n (M)	Times (hr) 1st/2nd	WAC (%)
CAH-1	90	E/E	0.25	24/24	2543
CAH-2	90	E/E	0.5	24/24	3342
CAH-3	50	E/E	0.25	24/24	908
CAH-4	50	E/E	0.5	24/24	1449

H_2O (3/2(v/v)) solution three times followed by three washes with IPA/deionized H_2O (3/1(v/v)). The film was immersed in IPA/deionized H_2O overnight. The film was allowed to dry in a fume cupboard.

2.4. Characterization of crosslinked HA

2.4.1. *Solid-state* ^{13}C-NMR *characterization*

The solid-state ^{13}C-NMR analysis of the uncrosslinked HA and double crosslinked HA was carried out at 50 MHz using an advance 200 spectrometer. The spectra were obtained using a contact time of 1 ms in the standard cross polarization (CP) pulse sequence.

2.4.2. *Rheological analysis*

The crosslinked HA gel was prepared by simply soaking into phosphate buffer solution (PBS) until fully swollen. The gel was collected and the residual water was carefully removed using a fiber-free paper. The HA concentration was analyzed using a Carbozol assay [20]. The rheological properties of uncrosslinked HA and crosslinked HA gel were analyzed using a T.A instruments CSL^2 500 Rheometer, fitted with a 4 cm 4° cone-plate geometer. All analyses were carried out at 25 °C.

2.4.3. *Water absorption capacity measurement*

20 mg of each dried crosslinked sample (W_d) were immersed in PBS solution for 24 h to obtain a fully swollen gel. The wet gel was filtered and the residual water at the surface removed using fiber-free tissue paper. The wet gel was weighed to obtain Ws. Thus the water absorption capacity (WAC %) was calculated according to formula (1):

$$\mathrm{WAC}\,(\%) = (W_s - W_d)/W_d \times 100 \qquad (1)$$

2.4.4. In vitro *biostability assessment of crosslinked HA*

2.4.4.1. Resistance to hyaluronidase. 20 mg of crosslinked HA was suspended in 6 ml PBS (pH = 7.4) containing 1000 U bovine testes hyaluronidase (EH3.2.1.35) and incubated at 37 °C for 24 h. The film was removed and rinsed using PBS. The rinse solution and incubation solution were made up to a known

volume and boiled for 30 min to precipitate the hyaluronidase. The solution was centrifuged and the supernatant made up to 25 ml using PBS. The HA concentration was measured using the Carbazole assay [20]. Hyaluronidase solution without crosslinked HA was selected as a control. The HA weight loss (%) due to hyaluronidase digestion was calculated using formula (2):

$$\text{HA weight loss}\,(\%) = [\text{HA}] \times 25/[\text{HA}]_0 \times 100 \quad (2)$$

in which, [HA] (mg/ml) is the concentration of HA, $[\text{HA}]_0$ is the original HA content (mg).

2.4.4.2. Resistance to free radicals. Fenton's reagents were used to create free radicals, which were formed by the addition of 25 µl 0.1 M H_2O_2 and 25 µl 0.1 M ascorbic acid to 5 ml PBS solution. The digestion was allowed to proceed for 24 h at 37 °C, after which, the film was removed and rinsed using PBS. The incubation and rinsing solutions were collected to obtain a 10 ml solution. The HA concentration was measured using the Carbazole assay [20]. Fenton's reagent/PBS solution without crosslinked HA was selected as a control. The HA weight loss (%) due to free radical degradation was calculated using the above formula (2).

3. Results and discussion

3.1. Synthesis of double crosslinked HA

The crosslinking of HA using epoxide was strongly dependent on the HA concentration, the pH value of the medium, reactant/HA feeding ratio, reaction temperature and the process itself. In particular the process conditions affect the biostability of the final products through variation in ester-linkage and ether-linkages. The first crosslinking, to form the stronger ether bond, was shown to be significant in determining the final biostability of the crosslinked product. The ether bonded HA is stable in acidic condition while the ester bonded HA is digested easily under the same conditions.

3.2. WAC % of crosslinked HA

As a hydrogel, the measurement of WAC of crosslinked HA is often used as an index for the degree of crosslinking. Normally, an increase in the degree of crosslinking will lead to a reduction in the WAC [9]. To evaluate the effectiveness of our double crosslinking technology, the water absorption capacity of crosslinked HA or HA/polymer was measured. Fig. 2 and Table II give the WAC (%) results of single crosslinked and double crosslinked samples.

Fig. 2 shows that the water absorption capacity is reduced after the second crosslinking of the single crosslinked HA. The same trend is observed when HA is combined with PVA. As shown in Table II, the single crosslinking can only produce either water-soluble products (CPH-5, 9, 10) or high water-uptake products (CPH-11 and 12). In contrast, double crosslinking can produce water insoluble hydrogels with varied water absorption capacities, which are related to the HA composition and crosslinking conditions. In comparison with their single crosslinked counterparts the water-uptake of the double crosslinked gels is considerably lower. The combination of HA and sodium alginate, was found to be influenced by the presence of calcium ions, which increased the water absorption capacity of the gel formed (Table III). The increase in calcium ion concentration is thought to reduce the reactivity of the carboxyl group due to ionic complexation, which may leave the carboxyl group free for absorbing more water (Table III).

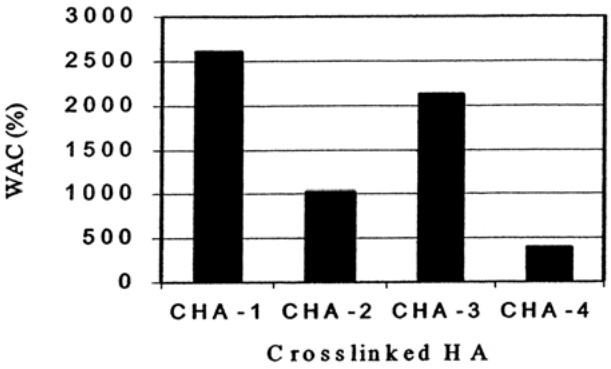

Figure 2 Water absorption capacity of crosslinked HA. CHA-1: single-crosslinked HA with epichlorohydrin; CHA-2: double-crosslinked HA based on CHA-1 using 1,2,7,8-diepoxyoctane; CHA-3: single-cross-linked HA with 1,2,7,8-diepoxyoctane; CHA-4: double-crosslinked HA based on CHA-3 using 1,2,7,8-diepoxyoctane.

3.3. Rheological studies

3.3.1. Shear rate dependence of viscosity (η, Pa)

The viscosity of uncrosslinked HA and crosslinked HA gel both are dependent on the shear rate. Fig. 3 gives the typical rheological profile of a pseudoplastic fluid for HA solution (1.5%). In contrast, crosslinked HA gel exhibits a tendency to an increased dynamic viscosity even at low frequency as shown in Fig. 4.

3.3.2. Shear Moduli (G′ and G″, Pa)

Elastic modulus G′ and the viscous modulus G″ of uncrosslinked HA and crosslinked HA gel were measured dynamically as shown in Figs 5 and 6. There is a crossover at 0.1 Hz for HA gel. It shows a viscous gel given $G'' > G'$ before this point and an elastic gel afterwards having $G' > G''$. For double crosslinked HA, the elastic modulus G′ is always greater than G″ and

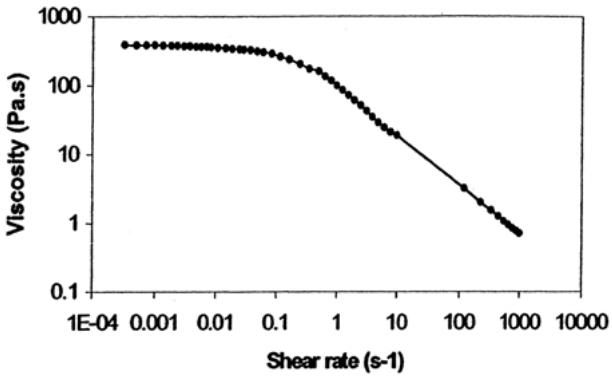

Figure 3 Viscosity dependence on shear rate (uncrosslinked HA with 1.5% concentration).

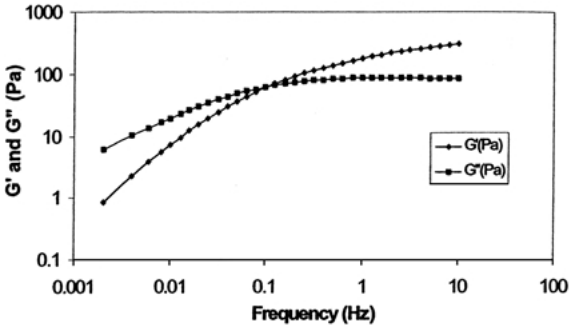

Figure 4 Rheological properties of HA gel (1.5%).

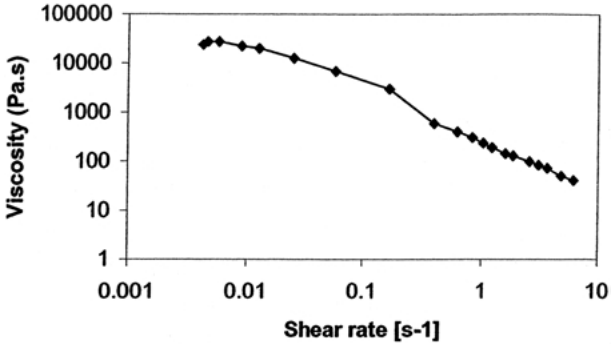

Figure 5 Viscosity dependence on shear rate (double crosslinked HA, with 1.6% HA).

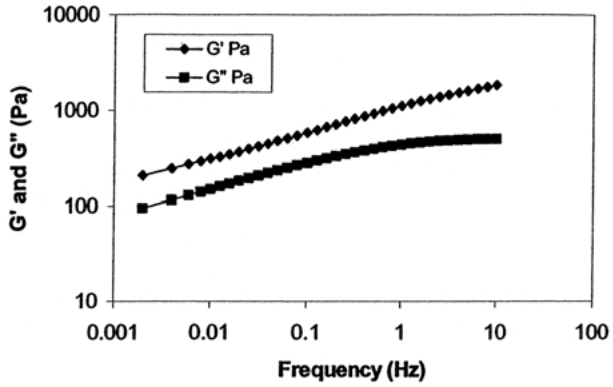

Figure 6 Rheological properties of double crosslinked HA.

there is no crossover point, which indicates the formation of an elastic gel network.

3.4. *In vitro* biostability study

The hyaluronidase and free radical digestion of crosslinked HA and HA/polymer were selected for determining the *in vitro* stability. The results shown in Figs 7 and 8 indicate that double crosslinking technology can produce a significantly more stable crosslinked HA network than that of single crosslinked sample. The resistance to hyaluronidase digestion is remarkably enhanced after the second crosslinking. The incorporation of other polymers within the HA network by this double crosslinking technology can produce materials with various physical properties and biodegradability as shown in Table III.

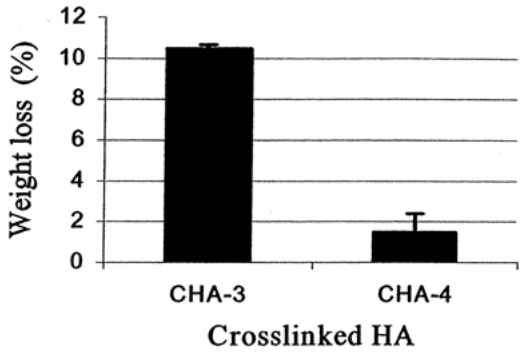

Figure 7 Resistance to hyaluronidase degradation.

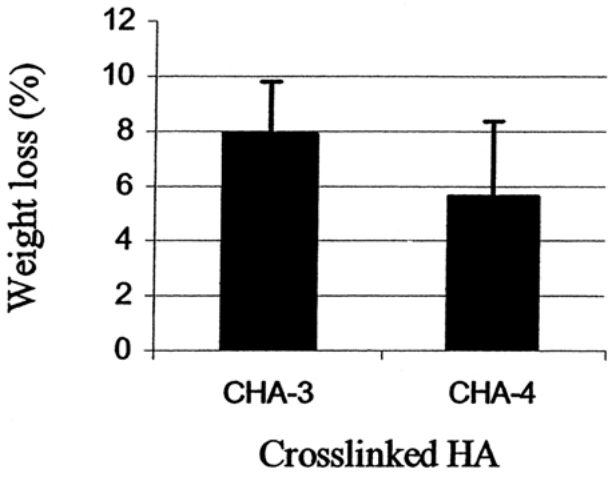

Figure 8 Resistance to free radical digestion.

3.5. Solid state ^{13}C-NMR characterization of crosslinked HA

Figs 9 and 10 depict the solid-state ^{13}C-NMR spectra of uncrosslinked HA and double crosslinked HA. The interpretation results are shown in Table IV.

Results reveal the presence of a long-chain methylene bridge (20–40 ppm) in the crosslinked HA network, which is formed during the crosslinking reaction. In addition, the chemical shift at 165–170 (shoulder) indicates the formation of an ester-linkage. It is very difficult to detect the formation of this bond by using other means [21].

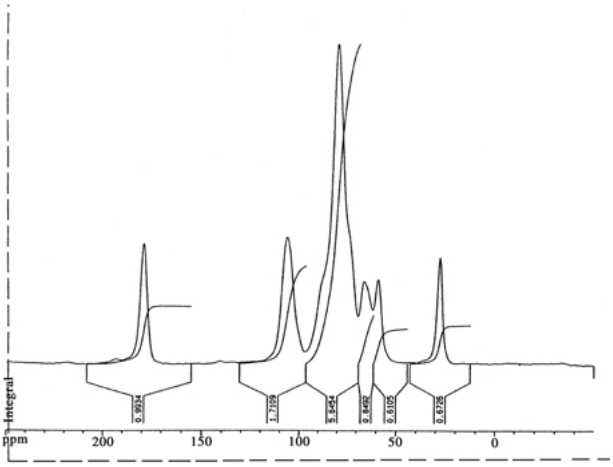

Figure 9 Solid state ^{13}C-NMR spectrum of HA.

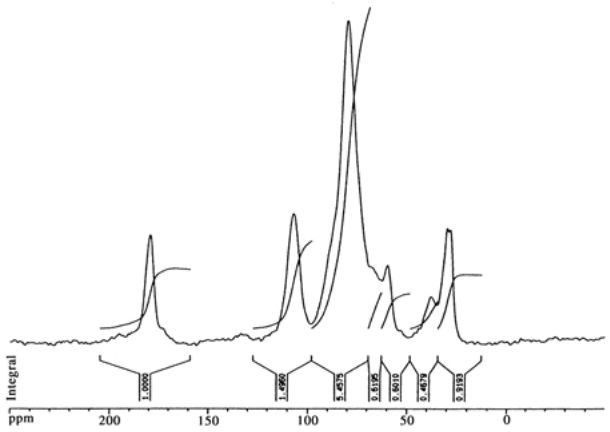

Figure 10 Solid state ^{13}C-NMR spectrum of crosslinked HA.

TABLE IV Summary of peak assignment of the solid ^{13}C-NMR spectra

Peak assignment	Chemical shift (ppm)
C=O in carboxyl and acetyl	170–180
C=O in ester	165–170
C_1	95–110
C_2–C_5 plus OCH and OCH2 for the ''bridge''	65–90
C_6	60–65
C–N	53–60
CH_2 in the ''bridge'' not bound to O	20–40
CH_3 in acetyl	20–25

4. Conclusions

This novel double crosslinking technology allows the production of various HA derivatives with varying degrees of crosslinking to give different physical properties. The technology has also been shown to be suitable for crosslinking HA with other polymers. We have shown using this novel process that hyaluronan whether crosslinked to itself or other polymers produces HA derivatives, which have a high degree of crosslinking and significantly improved biostability. Thus a range of water insoluble gels or films can be produced with properties tailored to meet the needs of their application. Biocompatibility testing of various biomaterials produced using this method is currently under investigation.

References

1. K. MEYER and J. W. PALMER, *J. Biol. Chem.* **107** (1934) 629.
2. K. P. VERCRUYSSE and G. D. PRESTWICH, *Crit. Rev. Ther. Drug. Carrier Syst.* **15** (1998) 513.
3. M. T. LONGAKER and N. ADZICK, *Clin. Mater.* **8** (1991) 223.
4. D. B. JOHNS, T. C. KIORPES, K. E. RODGERS and G. S. DIZEREGA *et al.*, *Fertility and Sterility* **68** (1997) 137.
5. F. DURANTI, G. SALTI, B. BOVANI, M. CALANDA and M. L ROSATI, *Derm. Surg.* **24** (1998) 1314.
6. E. BELL, *Tissue Eng.* **1** (1995) 163.
7. A. RASTRELLI, M. BECCARO, F. BIVIANO, G. CALDERINI and A. PASTORELLO, *Clin. Implant Mater.* **9** (1990) 199.
8. A. D. CAMPOCCIA, P. DOHERTY, M. RADICE, P. BRUN, G. ABATANGELO and D. F. WILLIAMS, *Biomaterials.* **19** (1998) 2101.
9. E. A. BALAZS and A. LESHCHINER, US Patent, 4,582,865, 1986.
10. N. YUI, T. OKANO and S. YASUHISA, *J. Control. Release.* **22** (1992) 105.
11. T. C. LAURENT, K. HELLSING and B. GELOTTE, *Acta. Chem. Scand.* **18** (1964) 274.
12. K. TOMIHATA and Y. IKADA, *J. Polym. Sci. Part A-Polym. Chem.* **35** (1997) 3553.
13. P. BULPITT and D. AESCHLIMANN, *J. Biomed. Mater. Res.* **47** (1999) 152.
14. K. TOMIHATA and Y. IKADA, *ibid.* **37** (1997) 243.
15. W. M. RHEE and R. A. BERG, US Patent 5,510,121, 1996.
16. A. N. DE BELDER and T. MALSON, US Patent 4,886,787, 1989.
17. K. T. DICKERSON, J. R. GLASS, L.-S. LIU, J. W. POLAREK, W. S. CRAIG, D. G. MULLEN and S. CHENG, US Patent 5,677,276, October 1997.
18. X. ZHAO, WO 00/46253, August 2000.
19. X. ZHAO, C. ALEXANDER and J. FRASER, WO 00/46254, August 2000.
20. T. BITTER and H. M. MUIR, *Anal. Biochem.* **4** (1962) 330.
21. K. TOMIHATA, Y. IKADA, *Biomaterials.* **18** (1997) 189.

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