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A novel hydrogel crosslinked hyaluronan with glycol chitosan

Wei Wang

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Abstract A novel hydrogel was prepared by crosslinking hyaluronan with glycol chitosan in aqueous solution using water soluble carbodiimide at nearly neutral pH and room temperature. The products can be easily formulated into injectable gels, various films, membranes and sponges for soft tissue augmentation, viscosupplementation, drug delivery, preventing adhesion of post operation, wound dressing and tissue engineering scaffolds. The said hydrogel has high water adsorption property and biostability. Rheololgical results of the gel showed a soft and viscoelastic structure. FTIR further confirmed the formation of amide bonds between carboxyl groups of hyaluronan and amine groups of glycol chitosan and no N-acylurea and other derivatives were identified.

Introduction

Hyaluronan (HA) is a naturally occurring linear polysaccharide composed of [23pc] alternating N-acetyl-D-glucosamine and D-glucuronic acid monosaccharide units linked with β-1,4-bonds and then the disaccharide units linked with β-1,3-glycoside bonds. HA is non-toxic, non-immunogenic and a very good bioresorbable polymer for biomedical and cosmetic applications [1–3]. However, HA is a water-soluble polymer and degraded and eliminated rapidly by oxidation and by enzymes, in particular hyaluronidase, when implanted or injected into a living body [1, 4, 5]. That limits its many potential applications. Chemical modification allows the physicochemical properties and residence time of hyaluronan to be tailored to specific needs while remaining its bioresorbability. Various attempts have therefore been made to prepare more stable forms of HA, especially by crosslinking HA molecules to themselves or other polymers.

There are three main approaches for chemically modifying hyaluronan to make water insoluble biomaterials with increasing biostability *in vivo*: (a) grafting pedants or side chains on hyaluronan macromolecules, (b) crosslinking hyaluronan by small molecular crosslinkers and (c) crosslinking hyaluronan with polymeric crosslinkers or coupling hyaluronan with other polymers using crosslinking agents.

Some small molecular chemicals have been reported to crosslink hyaluronan by approach (b), for instances, divinyl sulfone (DVS) [6], aldehydes such as formaldehyde [7], bi- or poly-functional epoxides such as 1,2,3,4-diepoxybutane and 1,4-butanediol diglycidyl ether (BDDE) [8, 9].

Through the approach (c) Balazs et al. [6] also disclosed crosslinking of hyaluronan with other biopolymers including xanthan gum, cellulose derivatives, collagen and heparin using DVS to prepare hydrogels. Dellavalle et al. [10] revealed the methods in which carboxyl polysaccharides such as hyaluronan, alginic acid, carboxymethycellulose and carboxymethychitin are crosslinked themselves or with each other by esterification. Rhee et al. [11] invented the method to crosslink glycosaminoglycan polymers, particularly deacetylated hyaluronan with collagen, using difunctionally activated PEG (polyethylene glycol) succinimidyl or PEG-propiondialdehyde or PEG-glycidyl diepoxide (molecular weight over a range of from about 100 to about 100,000). Luo et al. [12] reported crosslinking hyaluronan and ADH (adipic dihydrazide) derivative by PEG-propiondialdehyde (molecular weight: 3400).

W. Wang (✉)
Mentor Biopolymers Ltd Herriot Watt Research Park, Edinburgh EH14 4AP United Kingdom
e-mail: wwang@miswaco.com

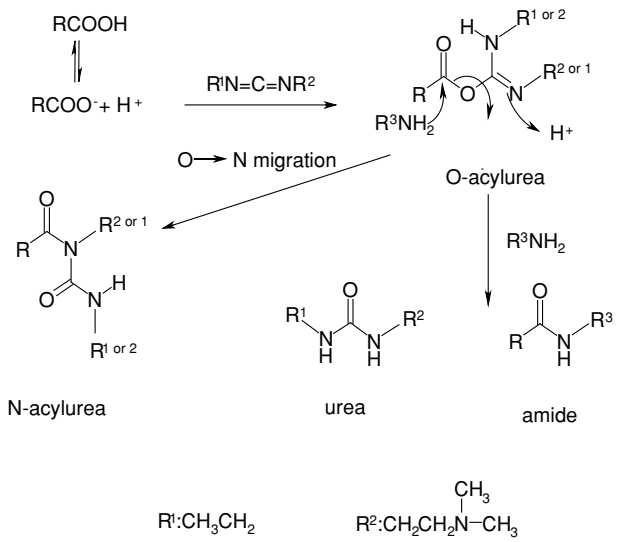

Fig. 1 Reaction mechanism of crosslinking HA with glycol chitosan using EDC

The use of water-soluble carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide methiodide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), to modify hyaluronan has been reported [13–15]. These EDC compounds can activate the carboxyl groups on HA in aqueous solution at pH 4.5 to form an O-acylurea carbodiimide-HA adduct (see Fig. 1), which is an unstable intermediate derivative. In the absence of a nucleophilic group, such as an amino acid or primary amine, it undergoes O–N migration to form the more stable N-acylurea adduct. However, in the presence of a nucleophile, preferably a primary amine R-NH_2, the predominant reaction is to form amide adducts between O-acylurea and R-NH_2 as well as a side product of water-soluble urea derivative by simple nucleophilic addition [1, 13–16].

Bulpitt et al. [16] reported that the carboxyl group of hyaluronan can couple with a monofunctional amine, a primary diamine and ADH by using EDC/HOBt (1-hydroxybenzotriazole) at pH 6.8 or EDC/NHS (N-hydroxysulfosuccinimide) at pH 7.5.

Glycol chitosan is a chitosan derivative containing amine groups along polymer chain and soluble in water at any pH. At lower pH solution glycol chitosan behaves a typical polycation due to the protonation of the amine groups [17]. Except for good biocompatibility and bioadhesion property, glycol chitosan has a much better biostability than hyaluronan [18, 19].

In this work a hydrogel was prepared for various biomedical and cosmetic applications by crosslinking HA with glycol chitosan in aqueous solution using water-soluble carbodiimide (EDC) at a pH range in which no polyelectrolyte complex forms between these two polyelectrolytes with opposite charges. Thus biostability of HA increased much and some new function are introduced, for example, amine groups are involved, which is useful for attachment and delivery of drugs, proteins and genes.

Experimental

Preparation of hydrogel

Hydrogels were prepared following different weight ratio of HA to glycol chitosan and varied molar ratio of EDC to carboxy group at different conditions such as pH, reaction time and salt concentration. An example is given below at a weight ratio of 1.8:1 (HA to glyol chitosan) and a molar ratio of 1.2:1 (EDC to HA) at room temperature for 3 h and without any salt added.

0.3 g solid hyaluronan (Mw: 2.06×10^6, provided by Vitrolife UK Ltd) was dissolved in 30 ml distilled water overnight at room temperature to obtain a homogeneous and clear solution (1%). 1.5 g glycol chitosan (Mw: 5×10^5, degree of deacetylation: 86%, Sigma) was dissolved in 100 ml distilled water overnight. The glycol chitosan solution (1.5%, pH: 9.1) was then filtered to remove any insoluble material. The pH value of the hyaluronan solution was adjusted to 4.0 by adding 0.1 M HCl under stirring. Then 13 ml of the glycol chitosan solution was added to the hyaluronan solution. After stirred for 30 min, 0.26 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide methiodide (Sigma) or 0.17 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (sigma) dissolved in 2 ml distilled water was added into the mixed solution under stirring and then kept stirring for designed time such as 3 h at room temperature. The formed hydrogel was then exhaustively washed with PBS (pH: 7.4) or distilled water to remove residual reagents and other small molecules.

Water absorption capacity

Approximately 20 mg of fully dried hydrogel sample obtained by drying purified hydrogel at 60°C under vacuum was immersed in 50 ml distilled water for 24 h, then the fully hydrated gels were filtered and removed residual water on the surface using tissue paper. The water absorption capacity (WAC) or swelling degree (SD) was calculated as follow,

$$WAC = (W - W_0)/W_0 \times 100\%$$

Where W_0 is the weight of initial dry sample and W is the weight of fully hydrated gel.

Chemical stability

The dried hydrogel samples were weighed and put in 6 M HCl aqueous solutions and 6 M NaOH aqueous solution respectively. At various intervals, sample dissolutions were observed and the residual samples were filtered and washed with water to neutral pH and then dried and weighed.

Rheological properties

Rheological properties of hydrogel were analysed using TA Instruments CSL 500 Rheometer fitted with a 4 cm and 4° cone-plate geometry. The measurements were carried out at 25°C.

FTIR spectroscopy

The purified hydrogel was cast onto glass petri dish and dried at room temperature. The FTIR spectra of the cast film were obtained using a Perkin-Elmer 1600 Series FTIR instrument. The specimen films were prepared by casting solutions of HA and glycol chitosan and their mixed solution containing EDC.

In vitro biostability

Resistance to hyaluronidase digestion

The prepared samples were sterilized at 121°C for 15 min and then dried by freezing drying and cut into small pieces. 20 mg was put in 6 ml PBS (pH: 7.4) containing 1000 U hyaluronidase (Sigma) and incubated in PBS solution at 37°C for 24 h. The solid was removed and rinsed with PBS. The rinsed and incubated PBS solution were put together and boiled for 15 min to precipitate hyaluronidase and then make up to 25 ml with PBS. Then opaque solution was centrifuged at 4000 RPM for 20 min and the clear supernatant was taken to measure hyaluronan concentration by carbozole assay. 6 ml PBS solution containing 1000 U hyaluronidase was used as a control. The hyaluronan weight loss was calculated following the formula as below,

$$\text{Hyaluronan weight loss} = [\text{HA}] \times 25/[\text{HA}]_0\} \times 100\%$$

Where [HA] (mg/ml) is hyaluronan concentration analysed by carbozole assay [20] and $[\text{HA}]_0$ is the original hyaluronan content (mg). carbozole was purchased from Sigma.

Resistance to lysozyme digestion [21.22]

Lysozyme (from egg white; Sigma), 15 mg, was dissolved in 500 ml of 0.1 M acetate buffer of pH 4.5. A ferricyanide solution was prepared by dissolving 0.5 g of potassium ferricyanide (Sigma) in 1000 ml of 0.5 M sodium carbonate.

25 mg samples sterilized at 121°C for 15 min and then dried by freezing drying was dispersed in 50 ml of 0.1 M acetate buffer (pH 4.5). 25 ml of the lysozyme solution was added, and the mixture was incubated at 37°C for 48 h. Then 3 ml of supernatant of the mixture was measured and 4 ml of the ferricyanide solution was added and boiled 15 min. It was cooled = with water for 5 min and the absorbance at 420 nm was measured using UV/Vis. Control reaction was carried out in the absence of lysozyme. Hydrolysis of the glycosidic linkages of glycol chitosan polymers was calculated as calibrated with N-acetylglucosamine (Sigma) of known concentration.

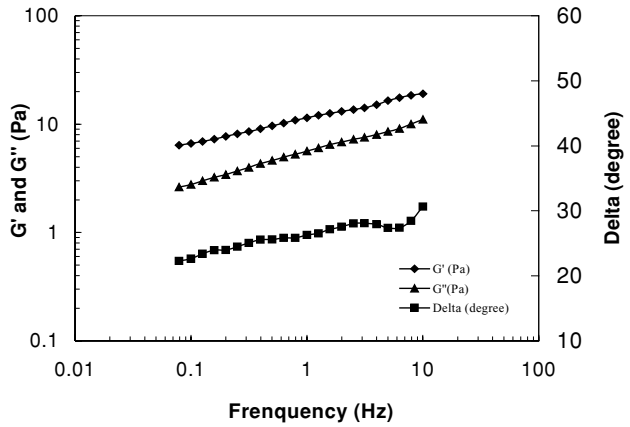

Fig. 2 Rheological properties of hydrogel crosslinked HA with glycol chitosan

Results and discussion

Hydrogel by crosslinking hyaluronan (HA) with glycol chitosan using EDC

HA is a polyanion, a polyelectrolyte complex can form and precipitate out of mixed solution if chitosan solution is added as chitosan is only soluble in acidic condition on which amine group of chitosan is protonated to amine ion [23, 24]. However, some chitosan derivatives such as glycol chitosan can dissolve at any pH water and at higher pH range the amine group cannot be protonated so that no complex forms between them and HA. It has been found in this work that by controlling pH value of mixed solutions, the formation of polyelectrolyte complexes can be avoided between HA and some chitosan derivatives. For example, if the pH of the resultant mixed solution is higher than 7.2 the formation of complex can be prevented. This can be confirmed by observation of the clear and transparent mixed solution, independent of whether the solutions are dilute or concentrated, and the

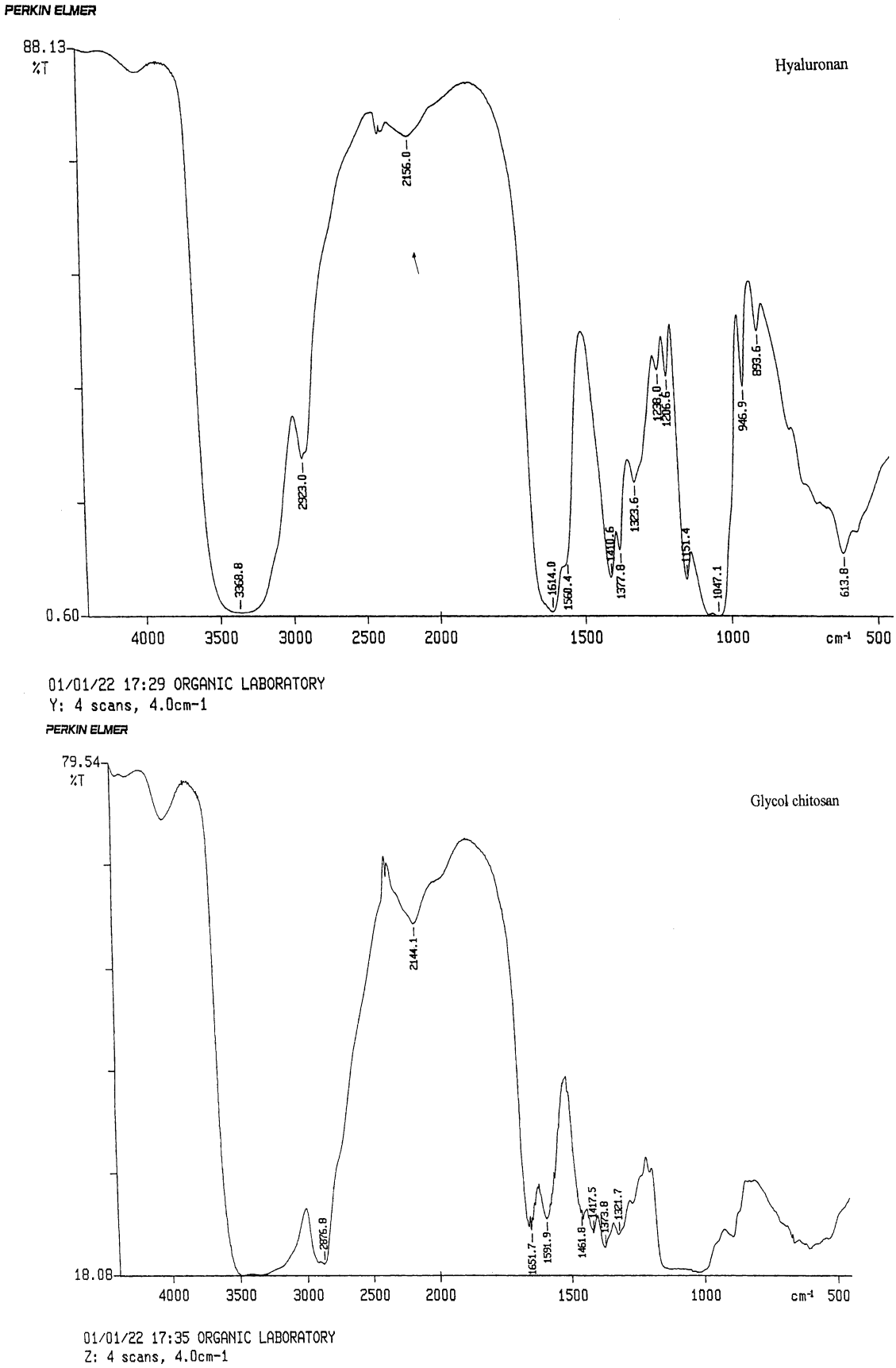

Fig. 3 FTIR spectrum of HA, glycol chitosan and their crosslinked hydrogel

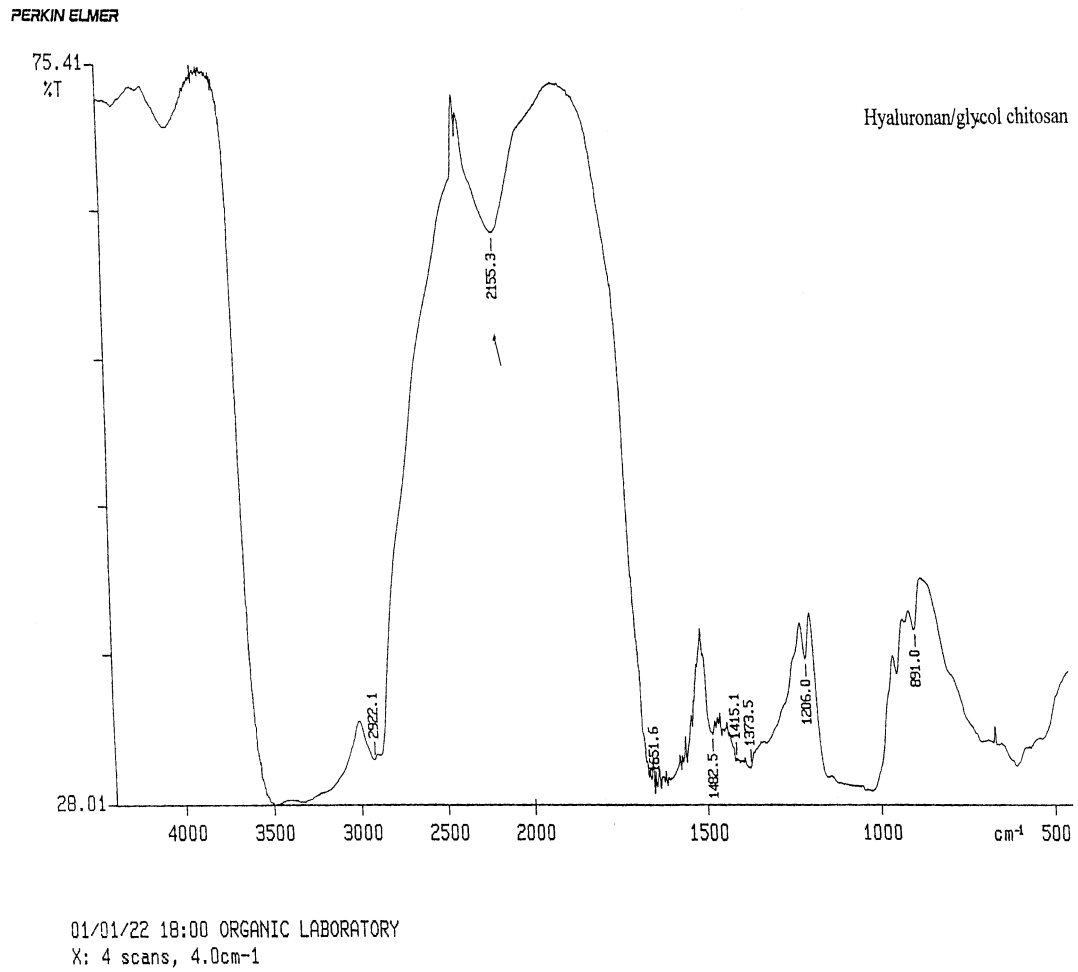

Fig. 3 Continued

complete dissolution of the dried cast film in distilled water. Also it was confirmed in this work that at nearly neutral pH around 7.5, carboxyl group of HA can be activated by EDC alone, no HOBt or HNS is needed [16].

The crosslinking reaction was carried out in 7.2–7.8 pH mixed solution of HA and glycol chitosan, particular at pH of 7.2-7.5, in which almost all the carboxyl groups of hyaluronan can dissociate and more than 80% of $–NH_2$ group of glycol chitosan cannot be protonated [19]. Therefore, no complex can form between them. Under such a condition, the carboxyl groups of hyaluronan can be activated by EDC, followed by nucleophilic reaction with $–NH_2$ groups of glycol chitosan in solution. During the reaction the mixed solution remains clear and the solution pH increases gradually up to about 9.0. At this pH, the degree of dissociation of $–NH_2$ group is almost 100%, so every $–NH_2$ group has the chance to react by nucleophilic addition to form amide bonds with carboxyl group, resulting in hyaluronan crosslinked with glycol chitosan.

The pH value of mixed solution of HA and glycol chitosan was controlled by adjusting pH of HA solution before mixing with glycol chitosan solution. As shown in Table 1, the mixed solution was cloudy and the complex precipitated if the mixed solution pH is lower than 7. If pH is higher than 7.2, a clear and transparent mixed solution was obtained, however, hydrogel cannot be obtained if pH is higher than 8 because carboxyl group cannot be activated at such a higher pH. Strong hydrogel formed within three hours at room temperature after EDC was added at pH of 7.2–7.5.

The reaction mechanism and product properties in this work are different from the work reported by Lee et al. [25]. In this patent they disclosed a method for forming hyaluronan covalently crosslinked with chitosan. The crosslinked product was formed in a soft solid complex state, through the amidation reaction in the presence of EDC. The amidation reaction was promoted by using the electrostatic attraction between the amine groups of the polymer and the carboxyl groups of the HA. And the crosslinking water insoluble materials have an opaque appearance and a low swelling degree of 100–500%. The amidation process is not 100% efficient and the maximum yield is 79%, so some polyelectrolyte complex containing ionic bonds remains at

Table 1 Effects of solution pH on the crosslinking reaction at 1.8:1 weight ratio of HA to glycol chitosan and 1.2:1 molar ratio of EDC to HA

Sample No.	1	2	3	4	5
pH of HA solution	3.5	4.1	5.2	6.4	7.5
pH of mixed solution with glycol chitosan	6.6	7.3	7.8	8.3	8.6
Apearance of mixed solution	complex	clear solution if pH is higher than 7.2			
Time of gelation	–	3 h	a week	none	none
Gel property	–	strong	weak	–	–
WAC (%)	–	5210	11050	–	–

Table 2 Effect of the ratio of hyaluronan/glycol chitosan on their crosslinking at 1.2:1 molar ratio of EDC to HA

Volume ratio (1%HA/1.5%GC)	10/5	10/8	10/7	10/10	5/10
Weight ratio (HA to GC)	2:1	1.8:1	1.5:1	1:1	0.5:1
Time of gelation	a week	3 h	3 h	a week	none
Gel property	weak	strong	strong	weak	–
WAC (%)	–	5530	5100	–	–

Table 3 Effect of salt concentration on crosslinking HA and glycol chitosan at 1.8:1 weight ratio of HA to glycol chitosan and 1.2:1 molar ratio of EDC to HA

Salt	NaCI			$CaCI_2$	
Concentration (%)	5%	10%	20%	10%	20%
Reaction time (H)	3	3	3	3	3
WAC (%)	4780	5210	5140	4950	5020

the end of the process. This has to be removed by an acid treatment.

Further, different weight ratios of HA to glycol chitosan were mixed for crosslinking and it was found that the crosslinking reaction rate is most fast at 1.5–1.8:1 weight ratio of HA to glycol chitosan. That is about 1:1 molar ratio of carboxyl group of HA to amine group of glycol chitosan. See Table 2.

Also the crosslinking rate increases with increasing added EDC amount, in this work most hydrogel was prepared following the 1.2:1 molar ratio of EDC to carboxyl group.

Salt was added up to 20% in mixed solution and found that salt does not obviously affect crosslinking of HA and glycol chitosan using EDC in solution (Table 3).

Hydrogel characterization

Typically the hydrogels have high water adsorption properties (4500–5500%). It is stable for weeks in 6 M HCI and 6 M NaOH respectively and no weight was lost. It was homogenized by mechanical treatment to microparticle. The resulting microgel was easily injectable through G30 Gauge needles. The polymer concentration was 1.3% measured by freeze-drying, very close to the theory concentration of 1.2 calculated from starting weight of HA and glycol chitosan used. Rheological measurement of sample 2 in the Table 1 is shown in Fig. 2. It indicates that the elastic modulus G′ (6–19 Pa) is higher than the viscous modulus G″ (2–11 Pa) and the delta is 20–30 over the frequency range of 0.1–10 Hz, meaning a very soft and elastic hydrogel.

Figure 3 shows FTIR spectra of HA, glycol chitosan and dried hydrogel film crosslinked HA and glycol chitosan (sample 2 in the Table 1). HA has a peak at 1614 cm^{-1} and a shoulder at 1560 cm^{-1}, which are assigned to amide I (C=O stretching) and amide II (N–H stretching) of N-acetyl amine group $–NHCOCH_3$ of HA), respectively. Glycol chitosan spectrum gives a peak at 1651.7 cm^{-1} assigned to amide bond of undeacetylated part of glycol chitosan (14%) and a peak at 1591.9 cm^{-1}, which is N–H bending of free amine group of glycol chitosan (deacetylated part). The hydrogel spectrum shows that a broad and strong peak at 1651.9 cm^{-1} and free amine peak at 1591.9 cm^{-1} decreased. This is because new amide bonds formed between HA and glycol chitosan and its adsorption peak overlapped with amide bond of $–NHCOCH_3$ of HA. It is also clear that a new peak at 1482.5 cm^{-1} appears which is assigned to C–N stretching of new amide bonds between HA and glycol chitosan.

Burns et al. [26] indicated by FTIR that absorption at 1700 cm^{-1} was found in all the films produced when no amine component has been added, which is assigned to N-acylurea adduct. However, in this work no peak was identified at 1700 cm^{-1}. Also no ester bond (1745–1760 cm^{-1}) and anhydride bond (1800 cm^{-1}) of carboxylic acid is observed on spectrum. These further confirmed that the main reaction was amidation between carboxyl group and amine group rather than N-acylurea addition of hyaluronan and EDC.

In vitro biostability

Digested with hyaluronidase (1000 units, Sigma) at 37°C for 24 h, the weight loss of the hydrogel sample 2 in Table 1 was 18%. The uncrosslinked HA degraded completely under the same condition. Only 0.08% of the glycosidic linkage of

glycol chitosan was hydrolysed after digestion with lysozyme at 37°C for 48 h. It is indicated that the hydrogel prepared in this work by crosslinking HA and glycol chitosan are very stable and should have longer lasting time if used in human body as medical devices

Conclusions

Carboxyl group of HA can be activated by water soluble carbodiimide EDC alone at pH of 7.2–7.8, at which condition no complex form between HA and glycol chitosan. A novel hydrogel can be thus prepared in solution by adjusting pH of HA solution before mixing it with glycol chitosan solution and the hydrogel products have very good and adjustable biostability.

References

1. G. D. PRESTWICK, D. M.MARECEK, J. F.MARECEK, K. P. VERCRUYSEE and M. R.ZIEBLL, in "The Chemistry, Biology and Medical Applications of Hyaluronan and Its Derivatives," edited by T. C. Laurent (Portland Press Ltd, London, 1998).
2. J. R. E. FRASER, T. C. LAURENT and U.B.G. LAURENT, *J. Int. Med.* **242** (1997) 27.
3. A. P. SCLAFANI and T. ROMO, *Facial Plastic Surgery* **16** (2000) 29.
4. K.TOMIHATA and Y. IKADA, *J. Biomed. Mater. Res.* **37** (1997) 243.
5. E. BELL, *Tissue Eng.* **1** (1995) 163.
6. E. A. BALAZES and A. LESHCHINER, US Patent 4582865, 1986.
7. E. A. BALAZS, A. LESHCHINER and P. BAND, US Patent 4713448, 1987.
8. T. LAURENT, K. HELLSING and B. GELOTTE, *Acta Chem. Scand.* **18** (1964) 274.
9. T. MAELSON and D. B. A. NORMAN, WO 86/00912, 1986.
10. F. DELLA VALLE and A. ROMEO, US Patent 5676964, 1997.
11. W. RHEE and R. A. BERG, US Patent 5510121, 1996.
12. Y. LUO and G. D. PRESTWICH, *J. Controlled Release*, **69** (2000) 169.
13. J.-W. KUO, D. A. SWANN and G. D. PRESTWICH, *Bioconjugate Chem.* **2** (1991) 232.
14. J.-W. KUO, D. A. SWANN, A. DAVID and G.D. PRESTWICH, US Patent 6537979, 2003.
15. R. HAMILTON, E. M. FOX, R. A. ACHARYA, A. RAKSHA and A. E. WALTS, US Patent 4937270, 1990.
16. P. BULPITT and D AESCHLIMANN, *J. Biomed. Mate. Res*, **47** (1999) 152.
17. K.SHINODA and A. NAKAJIMA, *Bull. Inst. Chem. Res.* **53** (1975) 400.
18. A. NAKAJIMA and K. SHINODA, *J. Appl. Polymer Sci.* **21** (2003) 1249.
19. L. MARTIN, K. F. WILSON, C.G, SENELS and I. F. UCHEGBU, *J. Control. Rel.* **80** (2002) 87.
20. T. BITTER and H. M. MUIR, *Analytical Biochem.* **4** (1962) 330.
21. T. IMOTO and K. YAGISHITA, *Agric. Biol. Chem.* **35** (1971) 1154.
22. K. KURITA, H. YOSHINO, S-I. NISHIMURA and S. ISHII, *Carbohydrate Polym.* **20** (1993) 239.
23. W. WANG, S. BO and W. QIN, *Int. J. Biolog. Macromolecules* **3** (1991) 281.
24. A. DENUZIERE, D. FERRIER and A DOMARD, *Carbohydrate polym.* **29** (1996) 317.
25. J.-Y. LEE, J-H. KIM and K.-B. HAN, WO.02.30990, 2002.
26. J. W. BURNS, R. MILLER and A. E. WALTS, US Patent 5527893, 1996.