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Research Article

Hyaluronic Acid Hydrogel Crosslinked with Complementary DNAs

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Hyaluronic acid (HA), a polysaccharide presents widely in the extracellular matrix of various animals, is used as an injectable gel material for regenerative medicine due to its biocompatibility. HA hydrogel can be produced from HA molecules via crosslinking. Physical crosslinking by noncovalent bonds is preferable rather than chemical crosslinking using a crosslinking agent to prevent unintended interactions in a biological environment and reduce inflammation caused by reaction by-products. In this study, HA was modified with two types of complementary single-stranded DNA obtained by digestion of pUC118 vector with the restriction enzyme Bsp1286I. When both of HA-DNAs were mixed, hybridized complementary strands acted as crosslinking points to form hydrogels. Shearing stress was applied to mix these DNA-conjugated HA solutions. As a result, a stiff hydrogel with an elastic modulus of about 100 Pa was obtained. The gel thus obtained would be facile to handle as an injectable gel that gains its structural properties from the shear stress applied through injection with a needle. In addition, DNA crosslinking points can be used for hybridization of the hydrogels with other biopolymers, cleavage with restriction enzymes and dissociation by thermal denaturation.

1. Introduction

Hydrogel is a three-dimensional network macromolecule with an ability to retain water molecules inside. Due to their excellent biocompatibility based on high water absorption and retention, hydrogels are widely used in medical applications, hygiene products and cosmetics. Hydrogels, prepared by chemical or physical crosslinking of natural polymers such as collagen and alginic acid, have been exploited in the fields of regenerative medicine and tissue engineering as scaffolds for cell adhesion, caging materials for cell printing and implantable materials. Hyaluronic acid (HA) is one of the most promising materials for hydrogel production in regenerative medicine [1].

HA, a biopolymer composed of D-glucuronic acid and D-*N*-acetylglucosamine linked via alternating β-1,4 and β-1,3 glycosidic bonds, is expressed ubiquitously as a glycosaminoglycan in the extracellular matrix (ECM) of various animals [2]. Unlike the proteins such as collagen, whose sequences differ from species to species, HA has low antigenicity and therefore is particularly expected to be applied as an injectable gel.

It has been shown that self-association of HA is induced by intermolecular interactions and entanglement of the biopolymer molecules [3–5]. However, to exploit HA as a hydrogel material, crosslinking is indispensable, because naturally occurring interactions strongly depend on the concentration and chain length of HA [6, 7]. Previously, various crosslinking chemistries, in which the functional groups were introduced into the carboxyl group of the side chain of HA, have been reported. In these reports, functional groups such as glycidyl methacrylic acid [8, 9], methacrylic acid [10], catecholamine [11], 2-dithiopyridyl group [12], and benzoylcysteine group [13] were used. However, chemical crosslinking might cause unintended interactions between the side chains and the biomolecules. Inflammation reactions might be caused due to reaction by-products and radicals. Furthermore, the process of gelation might be affected by the biological environment, such

as salt concentration, water content, temperature, and pH. For this reason, injectable gels for medical use should be crosslinked physically, but not by noncovalent bonds. For example, a polyion complex gel in which HA, a polyanion, electrostatically interacts with polycations, was previously reported [14–17]. There are also reports on gels where a peptide forming a β-sheet was used for crosslinking [18]. Physically-crosslinked hydrogel is advantageous because it can be feasibly prepared compared to chemically crosslinked gels by adjusting conditions such as salt concentration and pH; however it is difficult to precisely control the interaction. For this reason, physical gels where interactions of complementary DNA are used for crosslinking have been suggested and developed in recent years, such as peptide-DNA gel [19–21] and acrylamide-DNA gel [22, 23]. However, according to the latest reviews [24, 25], the physical gel of HA crosslinked with DNA has not been reported. DNA is a safe material featuring hydrophilicity and biocompatibility. Interaction specificity of complementary strands as well high specificity to enzymatic cleavage and linkage are also useful properties of DNA. Furthermore, the double-stranded DNA dissociates into two single strands with random coil conformation by heating. Since this process is reversible, sol–gel transition can be controlled by temperature.

In this study, we have focused on the advantages of interaction between DNA and fabricated HA modified with two single-stranded DNA linkers with complementary sequences. By mixing them and promoting hybridization between complementary strands, HA molecules can be crosslinked to each other. However, the interaction between short DNA strands (~20 bases) is weak [26] and might not be effective as a crosslinking point between HA molecules. Therefore, a long DNA chain of ~100 bases was used as a linker. However, it is difficult to obtain a large amount of long-chain DNA by chemical synthesis. Therefore, we obtained long DNA fragments by enzymatic treatment of DNA plasmid amplified in *Escherichia coli*.

2. Materials and Methods

2.1. Materials. Hyaluronic acid sodium salt from *Streptococcus zooepidemicus* (HA; FCH-200, MW 180 k–220 k): Kikkoman Corp. (Tokyo, Japan); competent cells (DH5α), pUC118 plasmid DNA, Bsp1286I, and T4 DNA ligase: Takara Bio Inc (Shiga, Japan); thiolated oligo DNAs with 5′ ends modified with C6 modifier (1-*O*-dimethoxytrityl-hexyl-disulfide-1′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Oligo1: thiol-5′-TTTTGTGCA-3′, Oligo2: thiol-5′-TTTTGTGCT-3′,)): synthesized and desalted by STAR Oligo of Rikaken Co., Ltd. (Nagoya, Japan); ethyl-3-(3-dimethylaminopropyl) cabodiimidehydrochloride (EDC): Dojindo Laboratories (Kumamoto, Japan); *N*-hydroxysulfosuccinimide sodium salt (NHS) and *N*-(2-aminoethyl) maleimide hydrochloride (AEM): Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); general reagents: Wako Pure Chemical Corp. (Osaka, Japan).

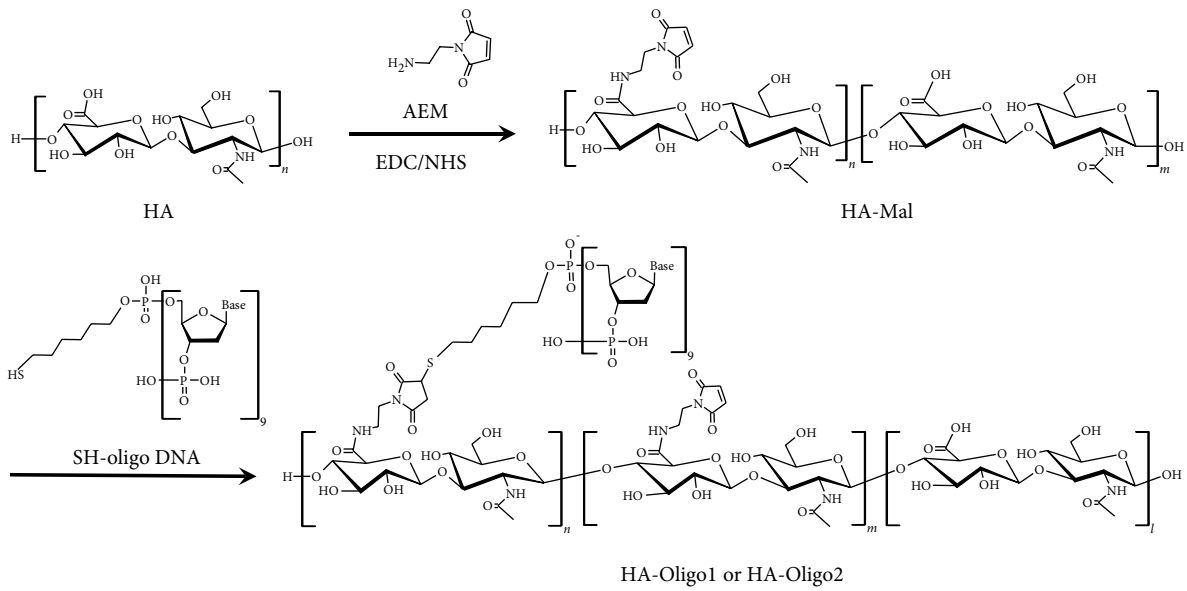
2.2. Preparation of Cleaved Plasmid DNA. According to the instructions of the manufacturer, pUC118 plasmid was transformed into competent cells using Ligation Mighty Mix. The plate culture was grown on LB medium with ampicillin (50 μg/mL) at 37°C, overnight. Plasmids were extracted from *Escherichia coli* using Genopure Plasmid Midi Kit (Roche), according to the instructions of the manufacturer. Plasmid DNA was precipitated with ethanol and then dissolved in water. Aliquots of plasmid DNA (1 μg) were incubated with Bsp1286I in the reaction buffer overnight at 30°C. After ethanol precipitation, it was dissolved in water to obtain cleaved plasmid DNA.

2.3. Modification of HA with Cleaved Plasmid DNA. Purchased thiolated oligo DNAs were incubated in 0.1 M dithiothreitol aqueous solution for 30 min for deprotection. Later the solution was loaded onto a reversed-phase chromatography column (Sep-Pak C1, Waters). The chromatography method included the following steps: equilibration and column wash with 2 M triethylamide acetate and elution with 30% acetonitrile. After the chromatography the eluted product was lyophilized. HA was dissolved in 50 mL of 0.1 M MES buffer (pH 6.0) to make HA concentration 0.2% (w/v). Next, 1.44 mg/mL of EDC and 0.86 mg/mL of NHS were added and the solution was mixed (30 min, RT). Further, 1.78 mg/mL of AEM was added and the mixture was incubated at room temperature overnight to obtain HA-Mal. After incubation, the solution was dialyzed (MWCO 10 kD) with 50 mM NaCl for 2 h and with distilled water for 48 h and then lyophilized. Deprotected HS-oligo DNA was added to 0.5% (w/v) HA-Mal aqueous solution to a final concentration of 30 μM and incubated (48 h, RT) to obtain HA-Oligo1 and HA-Oligo2. The product was dialyzed (MWCO 7 kD) with distilled water for 24 h and lyophilized to remove unreacted DNA oligomers completely. HA-Oligo1 and HA-Oligo2 were respectively dissolved in 600 μl of T4 ligase buffer to make a final concentration 0.5% (w/v) with 10 μg of cleaved plasmid DNA solution and 1050 units of T4 DNA ligase and incubated (1 h, 37°C). Then, an equivalent amount of 10 M urea solution was added, mixed by inversion and heated at 90°C for 10 min for denaturation. After quenching on ice, HA-Plasmid1 and HA-Plasmid2 were obtained, dialysed (MWCO 10 kD) with distilled water for 24 h and lyophilized.

2.4. Agarose Gel Electrophoresis. The samples were electrophoresed in 2% TAE agarose gel at 50 V for 1 h. Next, 50 bp DNA ladder (GeneDireX Inc.) was used as the molecular mass standard. The bands were stained with SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific) and visualised using the Gel Doc EZ Imager system (Biorad).

2.5. ^1H NMR. The ^{1}H NMR spectra were recorded to determine the detailed structures of the polymers using JEOL JNM-ECX500 (500 MHz) spectrometers. To set an accurate chemical shift, a coaxial insert (Wilmad-LabGlass) was used with the NMR tube for external locking (reference: $CHCl_3$ with 0.1% TMS).

2.6. Dynamic Viscosity Analysis. The frequency dependency of dynamic viscoelasticity was measured by a rheometer (Anton Paar, MCR 302/F). Three hundred microliters of the sample was sandwiched between cone plates (φ25 mm). The measurement was carried out at strain 1%, 37°C.



Scheme 1: Modification of hyaluronic acid with thiolated oligo DNAs.

3. Results and Discussion

3.1. Preparation of Oligo DNA-Modified HA. As shown in Scheme 1, the synthesis process of HA modification with oligo DNA was carried out by using carbodiimide [27]. The carboxyl group of glucuronic acid of **HA** was activated with EDC and NHS, maleimidized with *N*-(2-aminoethyl) maleimide hydrochloride (AEM) to obtain **HA-Mal** and coupled with thiolated oligo DNAs (Oligo1: thiol-5′-TTTTGTGCA-3′ and Oligo2: thiol-5′-TTTTGTGCT-3′). The ^{1}H NMR spectra of **HA**, **HA-Mal**, and the products **HA-Oligo1** and **HA-Oligo2** are shown in Figure 1. Based on the integral ratio of peaks derived from the acetyl group of HA (1.6 ppm) and the methyl group of thymidine of DNA (1.3 ppm), the degree of DNA substitution (DS) was calculated to be 4.7% and 5.6% for HA-Oligo1 and HA-Oligo2, respectively.

In a preliminary experiment, when HA modified with poly-A20 was mixed with HA modified with poly-T20, a gel was formed, but its mechanical strength was very weak (data not shown) due to weak A-T bonds. To make the interaction between the side chains robust and of high specificity, we extended the modified DNA with long complementary DNA strands. Scheme 2 shows a procedure for extending DNA chains and crosslinking of HA molecules. The reason for extending the DNA rather than directly modifying a synthesized long DNA strand on HA was due to the concern that the higher order structure formation in the DNA molecule reduces the yield after modification. Furthermore, the cost for the synthesis of long DNA fragments was also high.

Digested fragments of the plasmid pUC118 DNA were used to extend the modified DNA. Cleavage with Bsp1286I results in 4 base overhangs as shown in Scheme 3. Table 1 shows length and sequence details of 3′ overhangs for each fragment. Plasmid pUC118 DNA was cleaved with Bsp1286I almost completely (Figure 2, lane 2). When the cleaved DNA fragments were mixed with HA, the bands became smear and shifted to higher molecule weight (Figure 2, lane 4). The influence was particularly significant for larger size fragments. This result was observed due to the interaction between DNA molecules and the chains of HA.

As shown in Table 1, fragment F4 contains 85 bases and its 3′ overhang is complementary to Oligo1 and Oligo2. Therefore, F4 DNA fragments were ligated to HA-Oligo1 and HA-Oligo2 to obtain respective molecules modified with plasmid DNA fragments, HA-Plasmid1, and HA-Plasmid2. During the electrophoresis, the bands of HA-Plasmid1 and HA-Plasmid2 appeared as a smeared band corresponding to 50–100 bp molecular mass (Figure 2, lanes 5 and 6). The DNA is thought to be conjugated at multiple sites in one HA molecule; however it is difficult to observe it as a distinct band because it is single stranded.

3.2. Dynamic Viscoelasticity Measurement. To analyse the gelation process, viscoelasticity was measured by using the rheometer. Equal parts of 1% HA-Plasmid1 and 1% HA-Plasmid2 were put on a measurement plate of the rheometer and mixed with a micropipette. The dependency of the dynamic viscoelasticity on the frequency was measured. The solution (Figure 3(a)) turned into a gel after measurement (Figure 3(b)). When the measurement was repeated, a stiff gel was obtained (Figure 3(c)). The measurement was carried out three times consecutively. The frequency dependency measurement results of storage modulus and loss modulus are shown in Figure 4. Both HA and HA-Mal have shown frequency dependency which indicated sol-like behavior (Figure 4(a)). When the mixture of HA-Plasmid1 and HA-Plasmid2 was first analyzed, it has shown sol-like behaviour; however the change in storage modulus with respect to frequency was small during the second and the third measurement. After the third measurement, the storage modulus and tan δ of the gel were 55.5 Pa and 0.31, respectively, indicating that a network structure formed and a soft gel was obtained. An injectable

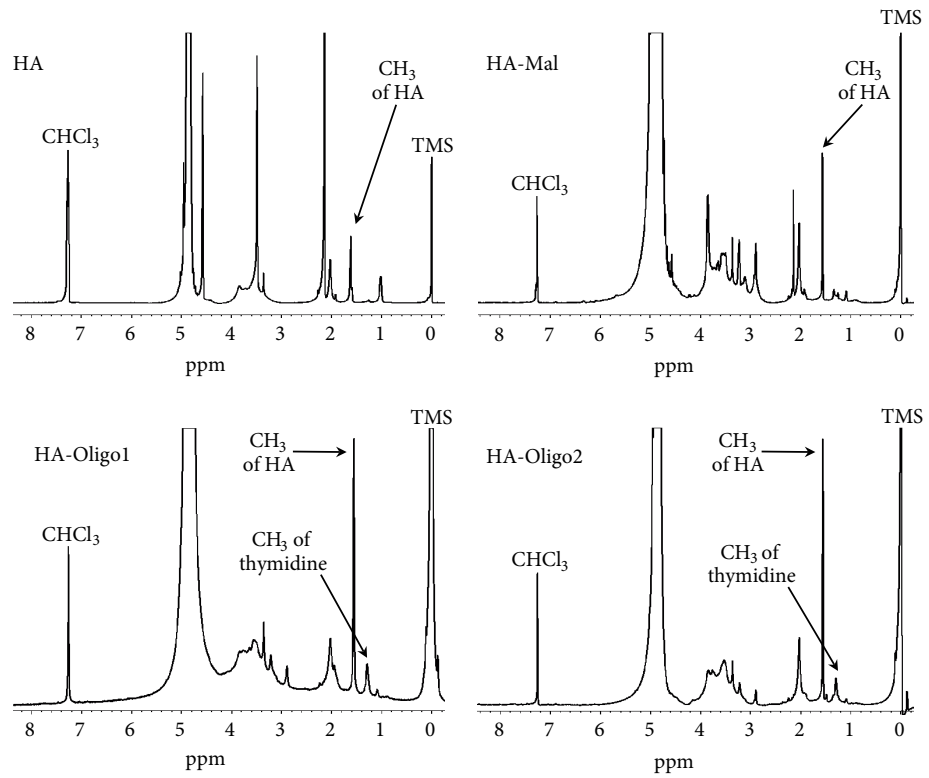
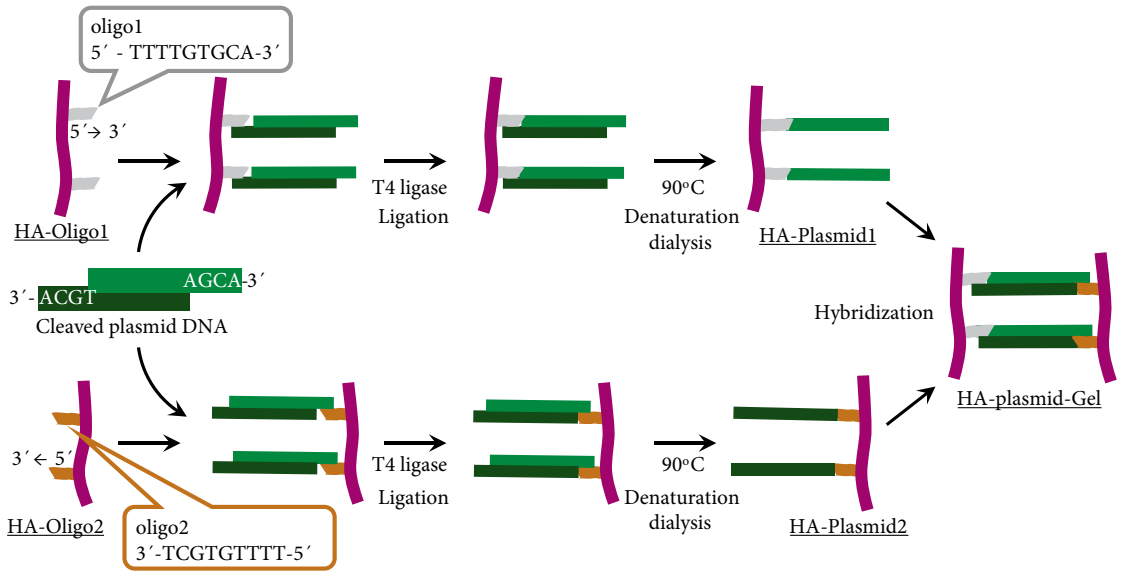


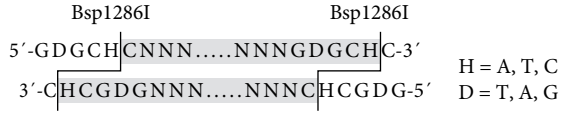
Figure 1: ^{1}H NMR Spectra of HA, HA-Mal, HA-Oligo1 (HA conjugated with Oligo1), and HA-Oligo2 (HA conjugated with Oligo2). Modification of hyaluronic acid with thiolated oligo DNAs.



Scheme 2: Schematic illustration of the crosslinking of HA-DNA via cleaved plasmid DNA fragments.

gel formed with two components usually forms immediately upon mixing. However, interestingly, our hydrogel formed only after shearing. DNA molecules would be not mixed and hybridized completely during the first measurement due to steric hindrance caused by the high molecular weight of HA-Plasmids. It is assumed that the shear stress applied during the measurement has facilitated the process of gel formation because the molecules were properly mixed, thus associated and hybridized with each other.

After the third measurement, aliquots of restriction enzyme Bsp1286I (1 μl) and 10 × L Buffer (15 μl) were added to 135 μl of the gel and the mixture was incubated overnight



Scheme 3: Cleavage site for Bsp1286I.

Table 1: DNA fragments of plasmid pUC118 DNA cleaved with Bsp1286I.

#	Cleavage position		Length	Sequences of sticky ends	
F1	241	679	438	5′-AGCT-3′	5′-GGCT-3′
F2	679	984	305	5′-AGCC-3′	5′-TGCA-3′[†]
F3	984	1481	497	5′-TGCA-3′[†]	5′-TGCA-3′[†]
F4	1481	1566	85	5′-TGCA-3′[†]	5′-AGCA-3′[‡]
F5	1566	2727	1161	5′-TGCT-3′	5′-TGCA-3′[†]
F6	2727	241	676	5′-TGCA-3′[†]	5′-AGCT-3′

[†]This sequence is complementary to 3′ end of DNA-a .[‡]This sequence is complementary to 3′ end of DNA-b.

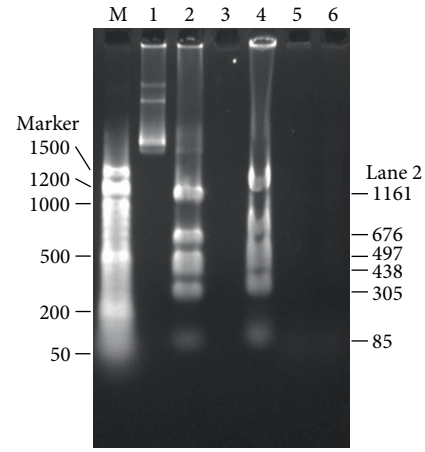


Figure 2: Electrophoresis of DNA samples. M: 50 bp DNA ladder; lane 1: pUC118 plasmid DNA; lane 2: cleaved plasmid DNA; lane 3: HA; lane 4: the mixture of HA and cleaved plasmid DNA; lane 5: HA conjugated with Plasmid1; lane 6: HA conjugated with Plasmid2.

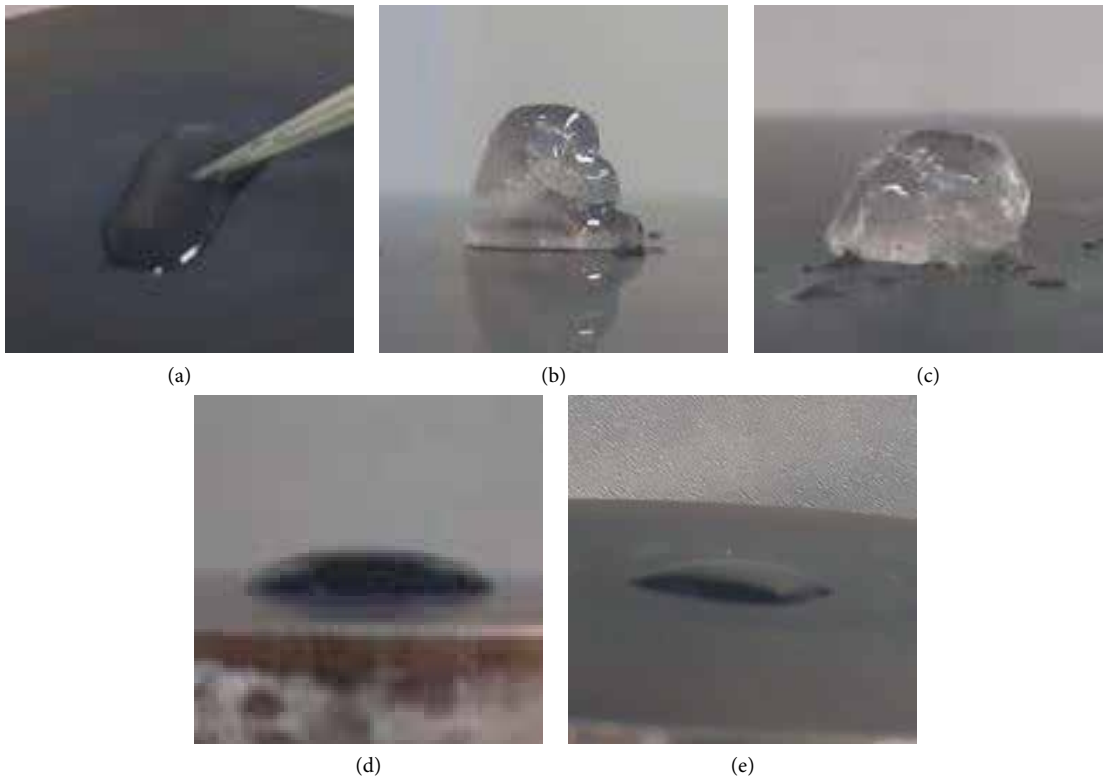


Figure 3: Macroscopic observation of the hydrogels on the plate during rheological analysis. (a–c) Mixture of HA-Plasmid1 and HA-Plasmid2. (a) Sample just after mixing was liquid. (b) Hydrogel was obtained after the first analysis. (c) Stiff hydrogel was obtained after the second analysis. (d) Native HA aqueous solution. (e) Hydrogel obtained after the third rheological analysis was treated with restriction enzyme. The sample changed into liquid.

at 30°C. After the incubation, rheological properties of the mixture were measured. As a result, the storage modulus has remarkably decreased to 0.7 Pa, but the elastic modulus did not depend on the frequency in the low frequency region (Figure 4(b)). This thixotropic property was caused by presence of the network structure aftesr the enzymatic treatment, although the cross-link point was disappeared.

4. Conclusions

Hydrogel are expected to be applied as regenerative medical materials due to their ability to absorb and retain water and their compositional similarity to living tissue. HA, one of the representative polysaccharides in living tissues, has excellent biocompatibility, thus it has a great potential to be used in

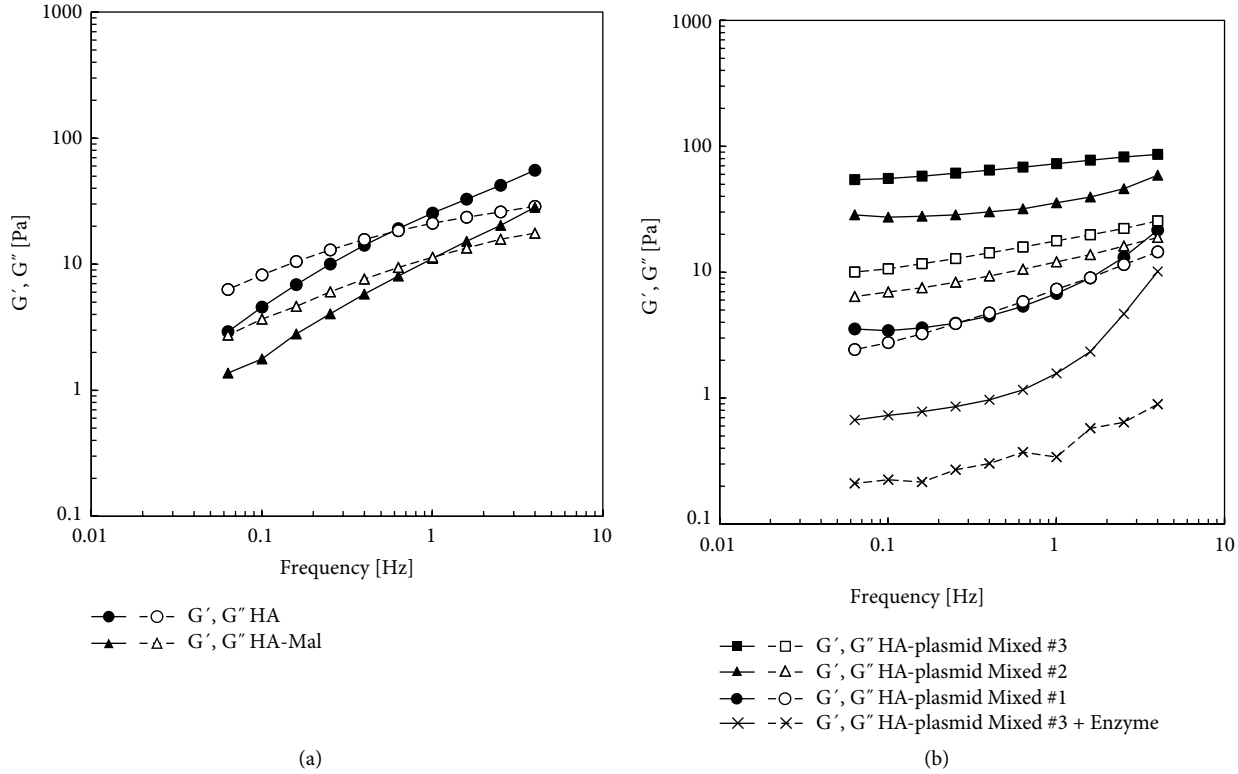


Figure 4: Frequency dependency of rheological properties. (a) Native HA solution and HA-Mal solution. (b) Mixture of HA-Plasmid1 and HA-Plasmid2. Measurements were repeated three times. Then, samples were treated with restriction enzyme (concentration: 1%; frequency: 0.05–5 Hz; strain: 1%; temperature: 37°C).

hydrogel production. However, HA molecules have to be crosslinked by addition of crosslinking agents or by introduction functional groups. Nevertheless, HA gelation with chemical crosslinkers makes the product difficult to use as an implantable material because it is difficult to control the reaction environment. In addition, there is a risk of cytotoxicity and inflammatory reactions, which can be caused by reactive residues and reaction by-products. Therefore, physically-crosslinked HA hydrogels are considered to be safer and more biocompatible.

In this study, HA was conjugated with DNA fragments obtained by the digestion of pUC118 plasmid with Bsp1286I. The hydrogel was formed due to DNA hybridization. By applying a certain shear stress, a relatively stiff gel, with elastic modulus of about 100 Pa, was obtained. The study shows that the injectable gel is easy to handle as it is a fluid which gelates by injection through a needle. In general, gels used as scaffolds for cell cultures have elastic modulus of about 30–300 Pa, thus we consider that the prepared HA hydrogel can be used as a cell scaffold. Gel stiffness can be controlled by changing the degree of maleimidization. Since the crosslinking occurs through hybridization of complementary DNA strands, such hydrogel can be modified by other biopolymers using the same hybridization technique. Such hydrogels can also be easily cleaved by restriction enzymes and dissociated by thermal denaturation. This property would also lead to the possibility to the application for the gene delivery. Thus, the material has a potential to biological and medical applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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